Sequence Detection Systems

Applied Biosystems 7900HT Real Time PCR System and 7300/7500 Real Time PCR Systems; ABI PRISM® 7000 Sequence Detection System and 7700 Sequence Detection System; GeneAmp® 5700 Sequence Detection System

Chemistry Guide

Applied Biosystems

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NOTICE TO PURCHASER:

PLEASE REFER TO THE *ABI PRISM® 7900HT Sequence Detection System and SDS Enterprise Database User Guide*, the *ABI PRISM® 7000 Sequence Detection System User Guide*, the *ABI PRISM® 7700 Sequence Detection System User's Manual*, the *GeneAmp® 5700 Sequence Detection System User's Manual*, the *Applied Biosystems 7300/7500 Real Time PCR System Relative Quantitation Getting Started Guide*, the *Applied Biosystems 7300/7500 Real Time PCR System Plus/Minus Getting Started Guide*, the *Applied Biosystems 7300/7500 Real Time PCR SystemAllelic Discrimination Getting Started Guide*, the *Applied Biosystems 7300/7500 Real Time PCR System Absolute Quantitation Getting Started Guide*, or the *Applied Biosystems 7300/7500 Real Time PCR System Installation and Maintenance Getting Started Guide* FOR LIMITED LABEL LICENSE OR DISCLAIMER INFORMATION.

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Preface

How to Use This Guide

Purpose of This Guide

The Applied Biosystems *Sequence Detection Systems Chemistry Guide* provides an easy-to-use reference on various techniques and applications, including:

- An introduction to sequence detection chemistries
- Background information, design guidelines, and general procedures for the following assay types:
  - Gene Expression and Quantification Assays
  - Allelic Discrimination Assays
  - Plus/Minus Assays
- Troubleshooting information

Audience

This guide is intended for users of Applied Biosystems Sequence Detection Systems instruments and chemistries who have a working knowledge of the polymerase chain reaction (PCR) process.

Text Conventions

This guide uses the following conventions:

- **Bold** indicates user action. For example:

  Type **0**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also used for emphasis. For example:

  Before analyzing, *always* prepare fresh matrix.
- A right arrow bracket (>) separates successive commands you select from a drop-down or shortcut menu. For example:

  Select **File > Open > Spot Set**.

  Right-click the sample row, then select **View Filter > View All Runs**.

User Attention Words

Two user attention words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below:

Note: Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! Provides information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

Examples of the user attention words appear below:

Note: The size of the column affects the run time.

Note: The Calibrate function is also available in the Control Console.

IMPORTANT! To verify your client connection to the database, you need a valid Oracle user ID and password.

IMPORTANT! You must create a separate Sample Entry Spreadsheet for each 96-well microtiter plate.

Safety Alert Words

Safety alert words also appear in user documentation. For more information, see "Safety Alert Words" on page xiv.

How to Obtain More Information

Related Documentation

When using this chemistry guide, you may find the following documents to be helpful references.

- *ABI PRISM® 7900HT Real Time PCR System and SDS Enterprise Database User Guide* (PN 4317596)
- *ABI PRISM® 7000 Sequence Detection System User Guide* (PN 4348266)
- *ABI PRISM® 7700 Sequence Detection System User's Manual* (PN 904989)
- *GeneAmp® 5700 Sequence Detection System User's Manual* (PN 4304472)
- *RQ Manager Software User Guide* (PN 4339753)
- *SNP Manager Software User Guide* (PN 4338776)
- *Primer Express Software Version 2.0 Users Manual* (PN 4329500)
- *Applied Biosystems 7300/7500 Real Time PCR System Relative Quantitation Getting Started Guide* (PN 4347824)
- *Applied Biosystems 7300/7500 Real Time PCR System Plus/Minus Getting Started Guide* (PN 4347821)
- *Applied Biosystems 7300/7500 Real Time PCR System Allelic Discrimination Getting Started Guide* (PN 4347822)
- *Applied Biosystems 7300/7500 Real Time PCR System Absolute Quantitation Getting Started Guide* (PN 4347825)
- *Applied Biosystems 7300/7500 Real Time PCR System Installation and Maintenance Getting Started Guide* (PN 4347828)

Note: For additional documentation, see "How to Obtain Support" on page xi.

Send Us Your Comments

Applied Biosystems welcomes your comments and suggestions for improving its user documents. You can e-mail your comments to:

techpubs@appliedbiosystems.com

How to Obtain Support

For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

In addition, the Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Safety and EMC Compliance Information

This section includes the following topics:

Safety Conventions Used in This Document xiv
General Instrument Safety xv
Chemical Safety xv
Chemical Waste Safety xvi
Biological Hazard Safety xviii

Safety Conventions Used in This Document

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word–**IMPORTANT, CAUTION, WARNING, DANGER**–implies a particular level of observation or action, as defined below:

Definitions

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

– Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

– Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

– Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Except for IMPORTANTs, each safety alert word in an Applied Biosystems document appears with an open triangle figure that contains a hazard symbol. *These hazard symbols are identical to the hazard icons that are affixed to Applied Biosystems instruments*.

Examples

The following examples show the use of safety alert words:

IMPORTANT! You must create a separate a Sample Entry Spreadsheet for each 96-well plate.

The lamp is extremely hot. Do not touch the lamp until it has cooled to room temperature.

CHEMICAL HAZARD. **Formamide**. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

ELECTRICAL HAZARD. Failure to ground the instrument properly can lead to an electrical shock. Ground the instrument according to the provided instructions.

General Instrument Safety

WARNING **PHYSICAL INJURY HAZARD.** Use this product only as specified in this document. Using this instrument in a manner not specified by Applied Biosystems may result in personal injury or damage to the instrument.

Chemical Safety

Chemical Hazard Warning

WARNING **CHEMICAL HAZARD.** Before handling any chemicals, refer to the Material Safety Data Sheet (MSDS) provided by the manufacturer, and observe all relevant precautions.

WARNING **CHEMICAL HAZARD.** All chemicals in the instrument, including liquid in the lines, are potentially hazardous. Always determine what chemicals have been used in the instrument before changing reagents or instrument components. Wear appropriate eyewear, protective clothing, and gloves when working on the instrument.

WARNING **CHEMICAL HAZARD.** Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

WARNING **CHEMICAL STORAGE HAZARD**. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

You can obtain from Applied Biosystems the MSDS for any chemical supplied by Applied Biosystems. This service is free and available 24 hours a day.

To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field, type in the chemical name, part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.

3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose
4. To have a copy of a document sent by fax or e-mail, select **Fax** or **Email** to the left of the document title in the Search Results page, then click **RETRIEVE DOCUMENTS** at the end of the document list.
5. After you enter the required information, click **View/Deliver Selected Documents Now**.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About MSDSs" on page xv.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Chemical Waste Safety

Chemical Waste Hazard

CAUTION **HAZARDOUS WASTE.** Refer to Material Safety Data Sheets and local regulations for handling and disposal.

WARNING **CHEMICAL WASTE HAZARD.** Wastes produced by Applied Biosystems instruments are potentially hazardous and can cause injury, illness, or death.

WARNING **CHEMICAL STORAGE HAZARD**. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).For additional safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

- Be aware that high solvent flow rates (~40 mL/min) may cause a static charge to build up on the surface of the tubing. Electrical sparks may result.

Biological Hazard Safety

General Biohazard

BIOHAZARD. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves. Read and follow the guidelines in these publications:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **http://bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**).

Additional information about biohazard guidelines is available at: **http://www.cdc.gov**

Introduction 1

This chapter covers:

Selecting an Assay Type 1-2
Selecting the Sequence Detection Chemistry 1-6
Selecting an Assay Source. 1-7
Performing the Assay 1-9
Selecting a Data Analysis Approach and Determining Results 1-9

Selecting an Assay Type

SDS Instruments

Applied Biosystems has four instruments in its Sequence Detection System (SDS) product line:

- Applied Biosystems 7900HT Real Time PCR System (7900HT System)
- Applied Biosystems 7300 Real Time PCR System (7300 System)
- Applied Biosystems 7500 Real Time PCR System (7500 System)
- ABI PRISM® 7000 Sequence Detection System (7000 System)
- ABI PRISM® 7700 Sequence Detection System (7700 System)
- GeneAmp® 5700 Sequence Detection System (5700 System)

Note: For more detailed information on these instruments, refer to the instrument user guides. See "How to Obtain More Information" on page x for a list of the user guide titles and part numbers.

Assay Types Supported

The three assay types (quantification, allelic discrimination, and plus/minus) can be divided into two categories, as shown in Table 1-1.

Table 1-1 Assay types

Real-Time PCR	For Information See	Endpoint Assay	For Information See
Quantification, including: • One-step reverse transcription polymerase chain reaction (RT-PCR) for RNA quantification • Two-step RT-PCR for RNA quantification • DNA quantification	"About Quantitative Assays" on page 3-4	Allelic Discrimination	"About Allelic Discrimination Assays" on page 4-2
		Plus/Minus	"About Plus/Minus Assays Using an IPC" on page 5-2

The sequence detection systems can be used to perform the assay types in Table 1-2.

Table 1-2 Sequence Detection Systems and Assay Types

Instruments	Real-Time Assays	Endpoint Assays	
	Gene Expression	Allelic Discrimination	Plus/Minus with IPC
7900HT System	Yes	Yes	No
7300 System	Yes	Yes	Yes
7500 System	Yes	Yes	Yes
7000 System	Yes	Yes	Yes
7700 System	Yes	Yes	Yes
5700 System[a]	Yes	No	No

a. The 5700 System cannot perform multiplex (“same-tube”) reactions; therefore, the 5700 System cannot be used for quantification assays using multiplex PCR, Allelic Discrimination Assays, or Plus/Minus Assays using an IPC.

Table 1-3 Consumable Types Supported

Instruments	Consumable		
	96-Well Micro-Plate	384-Well Plate	MicroFluidic Card (384)[a]
7900HT System	Yes	Yes	Yes
7300 System	Yes	No	No
7500 System	Yes	No	No
7000 System	Yes	No	No
7700 System	Yes	No	No
5700 System	Yes	No	No

a. For more information on the MicroFluidic card, see the *7900HT System User Guide*.

About Real-Time PCR Assays

Real-time PCR is the ability to monitor the progress of the PCR as it occurs. Data is collected throughout the PCR process rather than at the end of the PCR process.

In real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles.

About One-Step RT-PCR

RT-PCR is used to quantify RNA. RT-PCR can be performed as a one-step or two-step procedure.

The one-step RT-PCR performs RT as well as PCR in a single buffer system (Figure 1-1). The reaction proceeds without the addition of reagents between the RT and PCR steps. This offers the convenience of a single-tube preparation for RT and PCR amplification. However, the carryover prevention enzyme, AmpErase® UNG (uracil-N-glycosylase), cannot be used with one-step RT-PCR. In one-step RT-PCR, the presence of UNG would destroy the cDNA as it is being made. For information about UNG, see "Using UNG to Minimize Reamplification Carryover Products" on page 2-6.

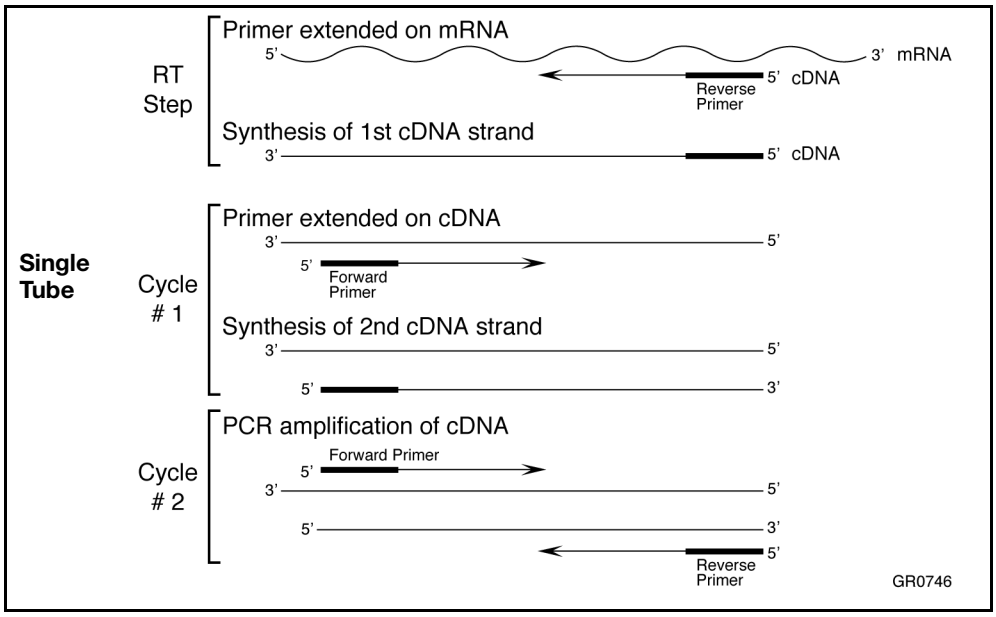


Figure 1-1 Schematic representation of one-step RT-PCR.

About Two-Step RT-PCR

Two-step RT-PCR is performed in two separate reactions (Figure 1-2). This is useful when detecting multiple transcripts from a single cDNA reaction, or when storing a portion of the cDNA for later use. When performing PCR, if dUTP is not used as a base in the RT step, AmpErase® UNG enzyme can be used to prevent carryover contamination.

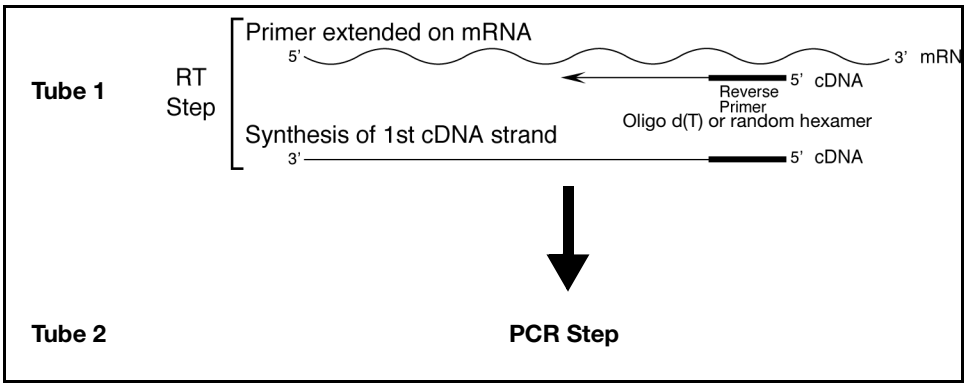


Figure 1-2 Schematic representation of two-step RT-PCR

Table 1-4 summarizes the differences between one- and two-step RT-PCR.

Comparison of RT-PCR Methods

Table 1-4 Primers for one- and two-step RT-PCR

Method	Primers for cDNA Synthesis	Features
One-step	Sequence-specific reverse primer	Requires single reaction mix UNG cannot be used
Two-step	Random hexamers	cDNA can be stored for later use UNG can be used Requires two reaction mixes
	Oligo $d(T)_{16}$	
	Sequence-specific reverse primers	

About Endpoint Assays

An endpoint assay (also called a plate read assay) measures the amount of accumulated PCR product in fluorescence units at the end of the PCR process. The datapoint is the normalized intensity of the reporter dye, or R_n.

Some endpoint assays can include both pre-PCR and post-PCR datapoints. In this case, the system calculates the delta R_n (ΔR_n) value per the following formula:

$$Rn\ post\text{-}PCR - Rn\ pre\text{-}PCR = DRn$$

About Multiplex PCR

Multiplex PCR is the use of more than one primer/probe set in the same tube. Multiplex PCR is most commonly used in 5′ nuclease quantification assays that involve relative quantification of gene expression.

Typically one probe is used to detect the target species; another probe is used to detect an endogenous control (internal control gene). Running both assays in a single tube reduces both the running costs and the dependence on accurate pipetting when splitting a sample into two separate tubes.

Selecting the Sequence Detection Chemistry

Applied Biosystems has developed two types of chemistries that can be used to detect PCR products on the SDS instruments:

- TaqMan® Probe-based fluorogenic 5′ nuclease chemistry
- SYBR® Green I dye chemistry

These chemistries are discussed in detail in Chapter 2.

TaqMan Probe-Based Chemistry

Applied Biosystems TaqMan Probe-based chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles. The Applied Biosystems patented fluorogenic probe design, which incorporates the reporter dye on the 5′ end and the quencher dye on the 3′ end, has greatly simplified the design and synthesis of effective 5′ fluorogenic nuclease assay probes (Livak, Flood, *et al*., 1995).

Assay Types that Use TaqMan Probe-Based Chemistry

The TaqMan Probe-based chemistry can be used for the following assay types:

- Quantification, including:
  - One-step RT-PCR for RNA quantification
  - Two-step RT-PCR for RNA quantification
  - DNA quantification
- Allelic Discrimination
- Plus/Minus

SYBR Green I Dye Chemistry

The SYBR Green I dye chemistry uses SYBR Green I dye, a double-stranded DNA binding dye, to detect PCR products as they accumulate during PCR cycles.

An important difference between the TaqMan probes and SYBR Green I dye chemistries is that the SYBR Green I dye chemistry will detect all double-stranded DNA, including nonspecific reaction products. A well-optimized reaction is therefore essential for accurate results.You can not perform multiplex PCR using SYBR Green I dye.

Assay Types that Use SYBR Green I Dye Chemistry

The SYBR Green I dye chemistry can be used for quantification assay types including:

- One-step RT-PCR for RNA quantification
- Two-step RT-PCR for RNA quantification
- DNA quantification

Selecting an Assay Source

After you select an assay type and sequence detection chemistry, you can:

- Purchase a predesigned assay (Assays-on-Demand™ Products)
- Have Applied Biosystems custom design an assay to your specifications (Assays-by-Design℠ Service)
- Design your own assay of TaqMan probe and primers, using Primer Express® software

Note: Assays-on-Demand Product and Assays-by-Design Service products are intended for use in singleplex reactions.

Assays-on-Demand Products

Assays-on-Demand products provide the most comprehensive collection of biologically informative, predesigned, quality-controlled, and validated assays ready to use on an ABI PRISM® Sequence Detection System.

Assays-on-Demand Products include:

- **Assays-on-Demand SNP Genotyping Products** (PN 4331183) – Assays for genotyping single nucleotide polymorphisms (SNPs). The products use the 5′ nuclease assay for amplifying and detecting specific SNP alleles in purified human genomic DNA samples. Each assay allows researchers to genotype individuals for a specific SNP.

  Designed using Applied Biosystems powerful bioinformatics pipeline and software, as well as genomic information from Celera Genomics and public databases. Each assay was validated on 90 genomic DNA samples to ensure the highest quality assay and to provide allele frequency information.
- **Assays-on-Demand Gene Expression Products** (PN 4331182) – Assays designed for the detection and quantification of specific nucleic acid sequences. These products provide researchers with optimized, ready-to-use 5′ nuclease assays for human and mouse transcripts. Gene expression quantification is performed in a two-step RT-PCR in which the PCR step is coupled with a 5′ fluorgenic nuclease assay.

For information on available products and specific product uses, contact your Applied Biosystems representative or visit the Applied Biosystems web site. See “How to Obtain Support” on page xi.

Assays-by-Design Service

Assays-by-Design service is an assay development service that designs, synthesizes, formulates, and delivers analytically quality-controlled primer and probe sets for SNP genotyping and gene expression assays based on sequence information submitted by the customer.

The following are Assays-by-Design service advantages:

- Use of the service allows researchers to save the time, money, and effort involved in assay design, synthesis, and formulation of the gene-specific assays.
- SNP genotyping assays are tested to reduce the need to test assays in your laboratory, saving you time and labor.

- Cost per data point is less than the cost of materials and time to "build-your-own."
- Assays are delivered ready-to-use: dilute the concentrated assay mix, add TaqMan Universal PCR Master Mix and cDNA, and load on an ABI PRISM Sequence Detection System platform of your choice.

For more information, contact your Applied Biosystems representative.

Designing Your Own Assay

When designing your own assay, follow the Assay Design Guidelines provided in this document. These simple, easy-to-follow guidelines have been developed by Applied Biosystems to optimize results when using SDS instruments and TaqMan probes or SYBR Green I dye chemistries.

Note: Applied Biosystems Assay Design Guidelines do not guarantee that all assays will provide the same level of performance and sensitivity. Even the most scrupulous design parameters cannot account for all the possible variables that can exist between two different assay systems.

Guidelines for quantification and allelic discrimination are detailed in "Designing Your Own Quantification Assay" on page 3-9 and "Designing Your Own Allelic Assay" on page 4-6.

Important Design Steps

Applied Biosystems Assay Design Guidelines contain the following important steps:

- Designing primers and probes using Primer Express software – The Primer Express software is used to design primers and probes. The software uses a set of default parameters to automatically select primer and probe sets.
- Selecting the appropriate reagent configuration – There are several TaqMan and SYBR Green I dye chemistry kits available. The reagent configuration you use will depend on your assay type. For quantification assays, see "Selecting the Appropriate Reagent Configuration" on page 3-13.
- **Using universal thermal cycling parameters** – All assay types designed using Applied Biosystems Assay Design Guidelines can be run using universal thermal cycling parameters. This eliminates any optimization of the thermal cycling parameters and allows multiple assays to be run on the same plate without sacrificing performance.
- **Using default primer and probe concentrations or optimizing primer and probe concentrations** – When you use Applied Biosystems Assay Design Guidelines, you can use default primer and probe concentrations for non-multiplex optimized assays or you can optimize primer and probe concentrations. For quantification assays, see "Optimizing Primer Concentrations" on page 3-17 and "Optimizing the Probe Concentration" on page 3-19.

IMPORTANT! These steps provide a rapid and reliable system for assay design and optimization only when used in their entirety. The system must be adopted as a whole in order to achieve the highest level of success, due to the interdependence of many of the individual components.

To illustrate this point, consider the following example. The ability to use universal thermal cycling parameters is based on the assumption that the selected primers have a melting temperature (T_m) of 58 to 60 °C as calculated by Primer Express software. If the primers do not have the correct T_ms, or even if the T_ms have been calculated with a primer design software package other than Primer Express, optimal performance and even functionality of the assay cannot be assured.

Visit the Applied Biosystems web site to access a variety of tutorials on how to use the Primer Express Software for designing real time quantitative assays. See "How to Obtain Support" on page xi.

Performing the Assay

For information about performing the assay on your system, see the documentation provided with your system. For information about laboratory practices, see "Minimizing DNA Contaminants" on page 2-6.

Selecting a Data Analysis Approach and Determining Results

For information, see Section 3.2, "Selecting a Data Analysis Approach and Determining Results."

Chemistry Overview 2

This chapter covers:

SYBR Green I Dye Chemistry 2-2
TaqMan Probe-Based Chemistry. 2-3
Selecting the Appropriate Chemistry 2-5
Minimizing DNA Contaminants 2-6

SYBR Green I Dye Chemistry

Development of SYBR Green I Dye Chemistry

Small molecules that bind to double-stranded DNA can be divided into two classes: intercalators and minor groove-binders (MGBs). Higuchi (Higuchi *et al.*, 1992) used the intercalator ethidium bromide for their real-time detection of PCR. Hoechst 33258 is an example of a minor groove-binding dye whose fluorescence increases when bound to double-stranded DNA (Higuchi *et al.*, 1993).

Regardless of the binding method, there are at least two requirements for a DNA binding dye for real-time detection of PCR products:

- Increased fluorescence when bound to double-stranded DNA
- No inhibition of PCR

Applied Biosystems has developed conditions that permit the use of the SYBR® Green I dye in PCR without PCR inhibition and with increased sensitivity of detection compared to ethidium bromide.

How the SYBR Green I Dye Chemistry Works

The SYBR Green I dye chemistry uses the SYBR Green I dye to detect PCR products by binding to double-stranded DNA formed during PCR. Here's how it works:

1. When SYBR Master Mix is added to a sample, SYBR Green I dye immediately binds to all double-stranded DNA.
2. During the PCR, AmpliTaq Gold® DNA Polymerase amplifies the target sequence, which creates the PCR product, or "amplicon."
3. The SYBR Green I dye then binds to each new copy of double-stranded DNA.
4. As the PCR progresses, more amplicon is created.

   Since the SYBR Green I dye binds to all double-stranded DNA, the result is an increase in fluorescence intensity proportional to the amount of double-stranded PCR product produced.

Figure 2-1 illustrates this process.

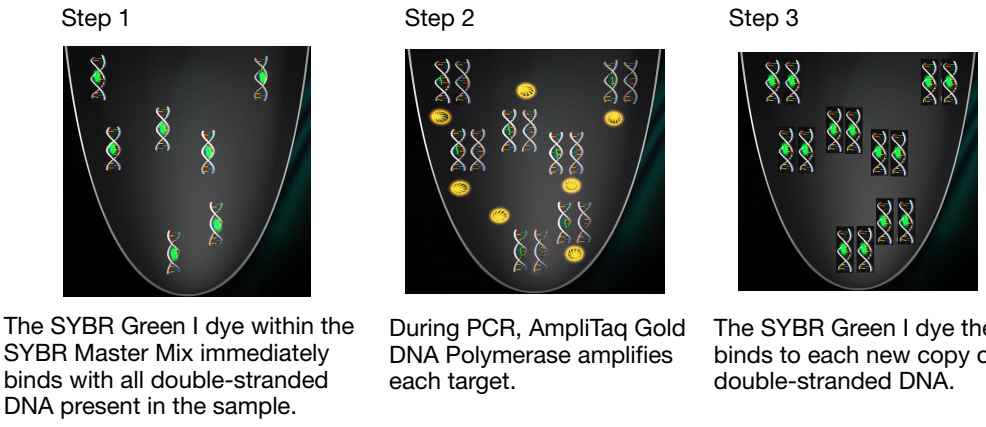

Step 1
Step 2
Step 3
The SYBR Green I dye within the SYBR Master Mix immediately binds with all double-stranded DNA present in the sample.
During PCR, AmpliTaq Gold DNA Polymerase amplifies each target.
The SYBR Green I dye then binds to each new copy of double-stranded DNA.

Figure 2-1 Representation of how the SYBR Green I dye acts on double-stranded DNA during one extension phase of PCR.

TaqMan Probe-Based Chemistry

Development of TaqMan Probe-Based Chemistry

Initially, intercalator dyes were used to measure real-time PCR products. The primary disadvantage to these type of probes is that they detect accumulation of both specific and nonspecific PCR products.

Real-time systems for PCR were improved by the introduction of fluorogenic-labeled probes that use the 5′ nuclease activity of AmpliTaq Gold DNA polymerase. The availability of these fluorogenic probes enabled the development of a real-time method for detecting only specific amplification products.

How TaqMan Sequence Detection Chemistry Works

The TaqMan® Probe-based chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR. Here's how it works:

1. An oligonucleotide probe is constructed with a fluorescent reporter dye bound to the 5′ end and a quencher on the 3′ end.

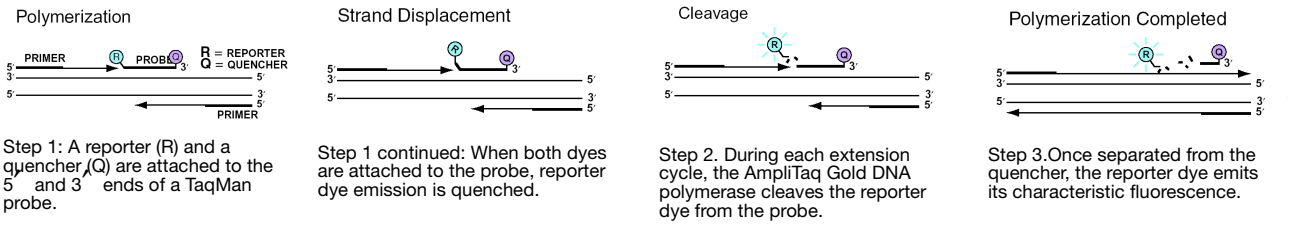
   While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET; Förster resonance, Förster, V. T. 1948) through space.

2. If the target sequence is present, the probe anneals between primer sites and is cleaved by the 5′ nuclease activity of AmpliTaq Gold polymerase during extension.

3. This cleavage of the probe:
   - Separates the reporter dye from the quencher, increasing the reporter dye signal.
   - Removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process.

4. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

Figure 2-2 illustrates this process.



Step 1: A reporter (R) and a quencher (Q) are attached to the 5′ and 3′ ends of a TaqMan probe.

Step 1 continued: When both dyes are attached to the probe, reporter dye emission is quenched.

Step 2. During each extension cycle, the AmpliTaq Gold DNA polymerase cleaves the reporter dye from the probe.

Step 3.Once separated from the quencher, the reporter dye emits its characteristic fluorescence.

Figure 2-2 Representation of how the 5′ nuclease chemistry uses a fluorogenic probe to enable detection of a specific PCR product.

Two Types of TaqMan Probes

Applied Biosystems offers two types of TaqMan probes:

- TaqMan probes with TAMRA™ dye as quencher
- TaqMan® MGB (minor groove-binder) probes with non-fluorescent-quencher (NFQ)

Table 2-1 describes each probe.

Table 2-1 Products

Custom Product	5′ Label	3′ Label	Other Features
TaqMan probe	FAM™, TET™, or VIC®	TAMRA	—
TaqMan MGB	FAM, TET, or VIC	Nonfluorescent quencher	minor groove-binder
Assays-on-Demand (TaqMan MGB)			
Gene Expression	FAM	Nonfluorescent quencher	minor groove-binder
SNP Genotyping	FAM, VIC	Nonfluorescent quencher	minor groove-binder
Assays-by-Design (TaqMan MGB)			
Gene Expression	FAM	Nonfluorescent quencher	minor groove-binder
SNP Genotyping	FAM, VIC	Nonfluorescent quencher	minor groove-binder

TaqMan MGB Probes Recommended

Applied Biosystems recommends the general use of TaqMan MGB probes, especially when conventional TaqMan probes exceed 30 nucleotides. The TaqMan MGB probes contain:

- **A nonfluorescent quencher at the 3′ end—**Allows the Real Time PCR instruments to measure the reporter dye contributions more precisely because the quencher does not fluoresce.
- **A minor groove-binder at the 3′ end—**Increases the melting temperature (T_m) of probes (Afonina *et al.*, 1997; Kutyavin *et al.*, 1997), allowing the use of shorter probes. Consequently, the TaqMan MGB probes exhibit greater differences in T_m values between matched and mismatched probes, which provides more accurate allelic discrimination.

Selecting the Appropriate Chemistry

The SYBR Green I dye and TaqMan Probe-based chemistry can be used for the assay types listed in Table 2-2. See Table 2-3 for considerations for choosing SYBR Green or TaqMan Probe-based chemistry.

Table 2-2 Assay type using SYBR Green 1 dye or TaqMan Probe-based chemistry

Chemistry	Assay Type		
	Quantification[a] (Chapter 3)	Allelic Discrimination (Chapter 4)	Plus/Minus using an IPC (Chapter 5)
SYBR Green I Dye	Yes	No	No
TaqMan probes	Yes	Yes	Yes

a. Includes one-step reverse transcription polymerase chain reaction (RT-PCR) and two-step RT-PCR for RNA quantification, and DNA/cDNA quantification

Table 2-3 Chemistry considerations for quantification assays using SYBR Green 1 dye or TaqMan Probe-based chemistry

Chemistry	Advantage	Limitation
SYBR Green I	• Reduces cost (no probe is needed) • Provides amplification of any double-stranded DNA sequence • Yields melting profile of distinct PCR products; allows melt curves to be added on the run • Increases sensitivity for detecting amplification products relative to product length	Can bind to nonspecific double-stranded DNA sequences. To avoid false positive signals, check for non-specific product formation using dissociation curve or gel analysis
TaqMan probe	• Increases specificity with a probe • Provides multiplex capability; allows for labelling probes with different, distinguishable reporter dyes, which allows detection of two distinct sequences in one reaction tube • Optimized assays available • Allows 5′ nuclease assay to be carried out during PCR • Provides combined specificity of primers and a probe • Requires specific hybridization between probe and target to generate fluorescent signal	

Minimizing DNA Contaminants

The DNA amplification capability of the PCR process makes special laboratory practices necessary when performing assays using fluorogenic 5′ nuclease (TaqMan Probe-based chemistry) or SYBR Green I dye chemistry. Potential contamination can be introduced by samples with high DNA concentrations, either from the DNA template controls or from PCR carryover.

In addition, due to the nonspecific nature of the SYBR Green I dye, any double-stranded DNA will be detected. When using the SYBR Green I dye chemistry, check for nonspecific product formation by using dissociation curve or gel analysis. Care must be taken to avoid contamination with target DNA. Gene expression assays that span exon-exon junctions minimize the effect of gDNA (genomic DNA) contaminants.

Note: The *ABI PRISM® 7700 Dissociation Curve Set-up Quick Start Guide* is available through the Applied Biosystems web site (see "How to Obtain Support" on page xi.)

Using UNG to Minimize Reamplification Carryover Products

AmpErase® uracil-N-glycosylase (UNG) is a 26-kDa recombinant enzyme encoded by the *Escherichia coli* uracil-N-glycosylase gene. This gene has been inserted into an *E. coli* host to direct expression of the native form of the enzyme (Kwok and Higuchi, 1989).

UNG acts on single- and double-stranded dU-containing DNA. It acts by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA (Longo *et al.*, 1990).

TaqMan Assays

For 5′ nuclease assays (which use the TaqMan Universal PCR Master Mix), AmpErase UNG treatment can prevent the re-amplification of carryover PCR products. When dUTP replaces dTTP in PCR amplification, AmpErase UNG treatment can remove up to 200,000 copies of amplicon per 50-µL reaction.

SYBR Green I Dye Assays

AmpErase UNG treatment can also be useful in preventing the re-amplification of carryover PCR products in SYBR Green I dye assays. Although the SYBR Green PCR Master Mix does not contain AmpErase UNG, dTTP has been replaced with dUTP, thus making the SYBR Green PCR Master Mix compatible with the use of AmpErase UNG. If contamination from PCR carryover is suspected, use AmpErase UNG to troubleshoot the problem.

Note: AmpErase UNG can be purchased individually (PN N808-0096) or as part of the SYBR® Green PCR Core Reagents kit (PN 4304886).

General PCR Practices

Use the following precautions to minimize sample contamination and PCR product carryover:

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification. Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup. Never bring amplified PCR products into the PCR setup area.
  - PCR amplification
  - Analysis of PCR products
- Open and close all sample tubes carefully. Avoid splashing or spraying PCR samples.
- Use positive-displacement or air-displacement pipettors with filter-plugged tips. Change tips after each use.
- Keep reactions and components capped as much as possible.
- Clean lab benches and equipment periodically with 10% bleach solution or 70% ethanol.

Gene Expression and Other Quantitative Assays

3

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Section 3.1 Introduction

In This Section

The following topics are covered in this section:

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About Quantitative Assays

What Is a Quantification Assay?

A Quantification Assay is a real-time PCR assay. It measures the amount of a nucleic acid target during each amplification cycle of the PCR. The target may be DNA, cDNA, or RNA.

There are three types of Quantification Assays discussed in this chemistry guide:

- DNA/cDNA quantification
- RNA quantification using one-step reverse transcription polymerase chain reaction (RT-PCR)
- RNA quantification using two-step RT-PCR

Note: For more information on one-step and two-step RT-PCR, see "About Real-Time PCR Assays" on page 1-3.

Instruments

Quantification Assays can be used with the following Sequence Detection System (SDS) instruments:

- Applied Biosystems 7900HT Real Time PCR System (7900HT System)
- Applied Biosystems 7300 Real Time PCR System (7300 System)
- Applied Biosystems 7500 Real Time PCR System (7300 System)
- ABI PRISM® 7000 Sequence Detection System (7000 System)
- ABI PRISM® 7700 Sequence Detection System (7700 System)
- GeneAmp® 5700 Sequence Detection System (5700 System)

IMPORTANT! The 5700 System cannot perform multiplex ("same-tube") reactions, so the 5700 System cannot be used for quantification assays using multiplex PCR.

Terms Used in Quantification Analysis

Terms commonly used in quantification analysis are defined in Table 3-1.

Table 3-1 Terms used in quantification analysis

Term	Definition
Amplicon	A short segment of DNA amplified during PCR.
Amplification plot	The graphical display of fluorescence signal versus cycle number.
Baseline	A line fit to the initial cycles of PCR, in which there is little change in fluorescence signal.
Threshold cycle (C_T)	The fractional cycle number at which the fluorescence passes the threshold.
Calibrator	A sample used as the basis for comparative results.
Endogenous control	Internal control gene present in each experimental sample. By using an endogenous control as an active reference, you can normalize quantification of a messenger RNA (mRNA) target for differences in the amount of total RNA added to each reaction.
Intron	A non-coding segment of gene.

Table 3-1 Terms used in quantification analysis *(continued)*

Term	Definition
No template control (NTC)	A sample that does not contain template. It is used to verify amplification quality.
Nucleic acid target (also called "target template")	Nucleotide sequence that you want to detect or quantify.
Passive reference	A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations caused by changes in concentration or volume. A passive reference dye is included in all SDS PCR reagent kits.
Reporter dye	The dye attached to the 5′ end of a TaqMan® probe. The dye provides a signal that is an indicator of specific amplification.
Normalized reporter (R_n)	The ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye.
Delta R_n (ΔR_n)	The magnitude of the signal generated by the given set of PCR conditions. The ΔR_n value is determined by the following formula: R_n – baseline
Standard	A sample of known concentration used to construct a standard curve. By running standards of varying concentrations, you create a standard curve from which you can extrapolate the quantity of an unknown sample. See "Relative Standard Curve Method for Quantification" on page 3-29.
Unknown sample	A sample containing an unknown quantity of template that you want to characterize.

Figure 3-1 shows a representative amplification plot and includes some of the terms defined above.

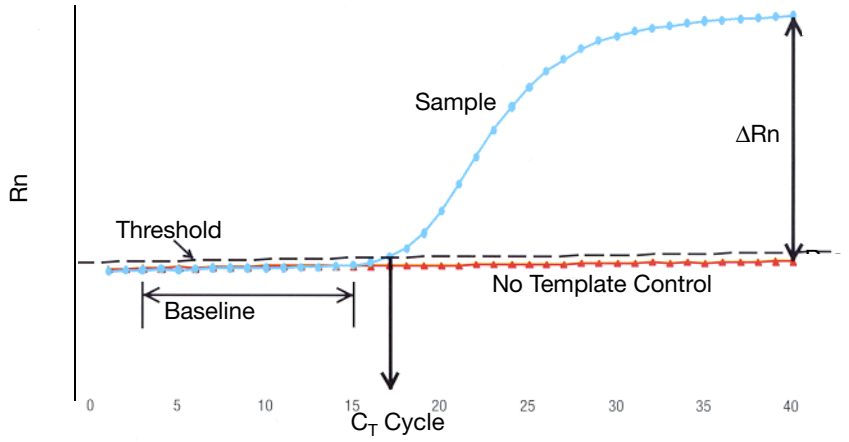

Figure 3-1 Model of a single-sample amplification plot, showing terms commonly used in quantitative analysis

How Real-Time PCR Quantification Assays Work

Real-time PCR allows reactions to be characterized by the point in time during cycling when amplification of a PCR product achieves a fixed level of fluorescence, rather than the amount of PCR product accumulated after a fixed number of cycles. An amplification plot graphically displays the fluorescence detected over the number of cycles that were performed.

As shown in Figure 3-1, in the initial cycles of PCR, there is no significant change in fluorescence signal. This predefined range of PCR cycles is called the “baseline”. First, the software generates a baseline subtracted amplification plot by calculating a mathematical trend using Rn values corresponding to the baseline cycles. Then, an algorithm searches for the point on the amplification plot at which the delta Rn value crosses the threshold. The fractional cycle at which this occurs is defined as the C_T.

Selecting a Quantification Assay Chemistry

Chemistries

Quantification Assays can be used with the following chemistries:

- TaqMan® Probe-based fluorogenic 5′ nuclease chemistry
- SYBR® Green I dye chemistry

Both TaqMan Probe-based and SYBR Green I dye chemistries can be used for either one-step or two-step RT-PCR.

Primers Used for cDNA Synthesis in Two-Step RT-PCR

For two-step RT-PCR, the following primers can be used for cDNA synthesis:

- Oligo $d(T)_{16}$
- Random hexamers
- Sequence-specific reverse primers

The choice of primers for reverse transcription is best made after experimentally evaluating all three priming systems. For short RNA sequences containing no hairpin loops, any of the three priming systems works equally well. For longer RNA transcripts or sequences containing hairpin loops, consider the guidelines in Table 3-2:

Table 3-2 Priming systems for RT-PCR

Primers	Selection Guidelines
Oligo $d(T)_{16}$	• Use to reverse transcribe only eukaryotic mRNAs and retroviruses with poly-A tails • Avoid long mRNA transcripts or amplicons greater than 2 kilobases upstream
Random hexamers	• Try first for use with long reverse transcripts or reverse transcripts containing hairpin loops • Use to transcribe all RNA (rRNA, mRNA, and tRNA)
Sequence-specific reverse primers	• Use to reverse transcribe RNA-containing complementary sequences only

Purchasing a Preformulated or Custom-Designed Quantification Assay

Assays-on-Demand Gene Expression Product

Product Description

Assays-on-Demand™ products for gene expression are biologically informative, preformulated gene expression assays that provide rapid, reliable results on human and mouse transcripts.

If the assay you need is not currently available, check the web site at a later time or use our Assays-by-Design℠ service (see below).

Purpose

Assays-on-Demand Gene Expression Products are designed for the detection and quantification of specific nucleic acid sequences. These products provide researchers with optimized, ready-to-use 5′ nuclease assays for human and mouse transcripts. Gene expression quantification is performed in a two-step RT-PCR in which the PCR step is coupled with a 5′ fluorogenic nuclease assay.

Product Properties

The following are properties of Assays-on-Demand Gene Expression Products:

- All Assays-on-Demand Gene Expression Products are designed and optimized to work with the TaqMan Universal PCR Master Mix, with or without AmpErase® UNG.
- A variety of targets and endogenous controls can be assayed in singleplex reactions in the same reaction plate with universal thermal cycling parameters.
- The Assays-on-Demand Gene Expression Products are designed to amplify target cDNA without amplifying genomic DNA. Assays denoted "m" in the assay name, for example, Hs00265057-m1, are designed so that the probe spans an exon-exon junction for multi-exon genes. Assays denoted "s" in the assay name, for example, Hs00257856-s1, are designed within an exon and, by definition, will detect genomic DNA.

Available Products

Assays-on-Demand Gene Expression Products (PN 4331182) consist of target assays and endogenous control assays to be used in a 5′ fluorogenic nuclease assay to amplify and detect expression of specific nucleic acid sequences. The latest information on available products and specific product uses is on the Applied Biosystems web site. See "How to Obtain Support" on page xi.

For more information about ordering Assays-on-Demand Gene Expression Products, contact your Applied Biosystems representative.

About Target Assays

Target assays from the Assays-on-Demand Gene Expression Products possess the following characteristics:

- A variety of Assays-on-Demand Gene Expression Products are available for detecting transcripts corresponding to many biological pathways.
- Many of the target assays are specifically designed to detect and quantify cDNA sequences without detecting genomic DNA.
- Target assays contain TaqMan MGB probes (FAM dye labeled) combined with primers at non-limiting concentrations.

Assays-by-Design Service

If a particular gene expression assay or SNP of interest is not available as an Assays-on-Demand product, Applied Biosystems Assays-by-Design service can design an assay based on sequence information you submit. The Assays-by-Design service (see "Selecting an Assay Source" on page 1-7) is an assay development service that designs, synthesizes, formulates, and delivers analytically quality-controlled primers and probes for gene expression assays.

For more information, contact your Applied Biosystems representative.

Designing Your Own Quantification Assay

Important Design Steps

This section discusses the Applied Biosystems Assay Design Guidelines developed specifically for Quantification Assays. These guidelines contain the following important steps:

- Designing primers and probes using Primer Express® software
- Selecting the appropriate reagent configuration
- Using universal thermal cycling parameters
- Using default primer and probe concentrations (or optimizing, if necessary)

IMPORTANT! These steps provide a rapid and reliable system for assay design and optimization only when used in their entirety. The system must be adopted as a whole in order to achieve the highest level of success, due to the interdependence of many of the individual components.

Conclusions

The Applied Biosystems Assay Design Guidelines enable Quantitative Assays to be designed and optimized rapidly and efficiently. Since thousands of assays have been developed this way, the following conclusions can be made.

- For the vast majority of 5′ nuclease quantification assays designed and run following these guidelines, using a concentration of 900-nM primers and 250-nM probe will provide for a highly reproducible and sensitive assay when using DNA or cDNA as a template.
- Due to the nonspecific nature of its detection, SYBR Green I dye primer optimization should be bypassed only with caution. However, if all guidelines are followed, concentrations of 50-nM forward and reverse primer should provide robust amplification with a good level of specificity when using DNA or cDNA as a template. This assumption should, however, always be verified by checking for nonspecific product formation with either dissociation curve or gel analysis.
- As a general rule, a 5′ nuclease quantification assay should enable detection and accurate quantification down to less than 50 copies of a target sequence, with even greater sensitivity possible.
- A SYBR Green I dye Quantification Assay is capable of similar performance; however, nonspecific product formation can potentially increase the minimum detection limit.

Primer and Probe Design Using Primer Express Software

The Primer Express software uses recommended parameters to select primers and probes based on the DNA sequence that you provide.

If you are designing your own assay, follow the summary of the primer and probe design guidelines for Quantitative Assays shown in Table 3-3 on page 3-12. For a detailed discussion of these guidelines see "About the Primer and Probe Design Guidelines" below.

Note: Even though no probe is required for SYBR Green I dye detection, it is still a good idea to use Primer Express software to select a primer and probe set when designing a SYBR Green I dye assay. Although no probe will be used, the primers will meet all the required criteria and if, in the future, there is the need to convert the assay to 5′ nuclease assay chemistry to obtain higher specificity, the probe can immediately be found in the original Primer Express software document.

Selecting an Amplicon Site for Gene Expression Assays

Selecting a good amplicon site ensures amplification of the target mRNA/cDNA without co-amplifying the genomic sequence, pseudogenes, and other related genes. SYBR Green I dye chemistry can be useful for screening amplicon sites for gene expression.

Guidelines

- The amplicon should span one or more introns to avoid amplification of the target gene in genomic DNA.
- The primer pair should be specific to the target gene to avoid amplification of pseudogenes or other related genes.
- When designing primers, use Primer Express guidelines.
- If no good sequence is found, it may be necessary to examine the sequence and redesign the amplicon or simply screen for more sites.

If the gene you are studying does not have introns, then it is not possible to design an amplicon that will amplify the mRNA sequence without amplifying the gene sequence. In this case, it is necessary to run RT minus controls.

About the Primer and Probe Design Guidelines

Selection of Small Amplicons

An important default parameter in Primer Express software is the selection of amplicons in the 50- to 150-basepair range. Small amplicons are favored because they promote high-efficiency amplification.

In addition, high-efficiency assays enable relative quantification to be performed using the comparative C_T method $(\Delta\Delta C_T)$ (Livak and Schmittgen, 2001). This method increases sample throughput by eliminating the need for standard curves when looking at expression levels of a target relative to a reference control. (For more information on the comparative C_T method, see page 3-33.)

G/C Content

Whenever possible, primers and probes should be selected in a region with a G/C content of 30 to 80%. Regions with a G/C content in excess of this may not denature well during thermal cycling, leading to a less efficient reaction. In addition, G/C-rich sequences are susceptible to nonspecific interactions that may reduce reaction efficiency and produce nonspecific signal in SYBR Green I dye assays. For this same reason, primer and probe sequences containing runs of four or more G bases should be avoided.

Melting Temperature

Selecting primers and probes with the recommended melting temperature (T_m) allows the use of universal thermal cycling parameters. Having the probe T_m be 8 to 10 °C higher than that of the primers is recommended.

5′ End of Probes

Primer Express software does not select probes with a G on the 5′ end. The quenching effect of a G base in this position will be present even after probe cleavage. This can result in reduced fluorescence values (ΔR_n , see Table 3-1 on page 3-4), which can impact the performance of an assay. Having G bases in positions close to the 5′ end, but not on it, has not been shown to compromise assay performance.

3′ End of Primers

The last five bases on the 3′ end of the primers should contain no more than two C and/or G bases, which is another factor that reduces the possibility of nonspecific product formation. Under certain circumstances, such as a G/C-rich template sequence, this recommendation may have to be relaxed to keep the amplicon under 150 basepairs in length. In general, avoid primer 3′ ends extremely rich in G and/or C bases.

Summary of Primer and MGB Probe Design Guidelines

Table 3-3 summarizes primer and probe guidelines for MGB probe design.

Table 3-3 Primer and probe design guidelines for quantitative assays

<table>
<tr><th>Probe Guidelines</th><th>Primer Guidelines</th></tr>
<tr><td colspan="2">Select the probe first and design the primers as close as possible to the probe without overlapping it (amplicons of 50 to 150 basepairs are strongly recommended).</td></tr>
<tr><td colspan="2">Keep the G/C content in the 30 to 80% range.</td></tr>
<tr><td colspan="2">Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.</td></tr>
<tr><td>When using Primer Express software, the T_m should be 68 to 70 °C.</td><td>When using Primer Express software, the T_m should be 58 to 60 °C.</td></tr>
<tr><td>No G on the 5′ end.</td><td rowspan="3">The five nucleotides at the 3′ end should have no more than two G and/or C bases.</td></tr>
<tr><td>Select the strand that gives the probe more C than G bases.</td></tr>
<tr><td>Make TaqMan MGB probes as short as possible, without being shorter than 13 nucleotides.</td></tr>
</table>

Selecting the Appropriate Reagent Configuration

There are several TaqMan Probe-based and SYBR Green I dye chemistry kits available for Quantitative Assays. The reagent configuration you use depends on your assay type.

The recommended reagent configurations are listed in the tables below. A detailed discussion of the primary reagents follows on page 3-14.

Recommended Reagent Configurations

Note: The part numbers listed below are for 200 reactions. See Appendix C for a list of available kit sizes.

A number of different reagents are available for DNA and cDNA Quantification Assays.

Table 3-4 Reagents available for DNA and cDNA quantification assays

Chemistry	Reagent Configuration	Part Number
TaqMan probes	TaqMan Universal PCR Master Mix	4304437
	TaqMan Universal PCR Master Mix, No AmpErase UNG	4324018
	TaqMan PCR Core Reagents Kit	N808-0228
SYBR Green I dye	SYBR Green PCR Master Mix	4309155
	SYBR Green PCR Core Reagents	4304886

RNA Quantification Using One-Step RT-PCR

A number of reagents are available (Table 3-5) for RNA Quantification Assays using one-step RT-PCR.

Table 3-5 Reagents available for RNA quantification assays using one-step RT-PCR

Chemistry	Reagent Configuration	Part Number
TaqMan probes	TaqMan One-Step RT-PCR Master Mix Reagents Kit	4309169
	TaqMan Gold RT-PCR Kit (without controls)	N808-0232
	TaqMan EZ RT-PCR Core Reagents **Note:** Use this configuration when a high-temperature RT step is required.	N808-0236
SYBR Green I dye	SYBR Green RT-PCR Reagents	4310179

RNA Quantification Using Two-Step RT-PCR

A number of reagents are available (Table 3-6) for RNA Quantification Assays using two-step RT-PCR.

Table 3-6 Reagents available for RNA quantification assays using two-step RT-PCR

Chemistry	Step	Reagent Configuration	Part Number
TaqMan probes	PCR step only	TaqMan Universal PCR Master Mix	4304437
	RT step only	TaqMan Reverse Transcription Reagents	N808-0234
		High Capacity cDNA Archive Kit	4322171
	Both RT and PCR steps	TaqMan Gold RT PCR kit	N808-0232
SYBR Green I dye	PCR step only	SYBR Green Master Mix	4309155
	Both RT and PCR steps	SYBR Green RT-PCR Reagents	4310179

About Universal Master Mix Reagents

TaqMan Universal PCR Master Mix

The TaqMan Universal PCR Master Mix is a reagent specifically designed to provide optimal performance for 5′ nuclease assays that use cDNA or DNA as a template. This product contains components that ensure excellent assay performance even when demanding G/C-rich target sequences are encountered. The use of one reagent for all assays simplifies the process of assay implementation.

SYBR Green I PCR Master Mix

The SYBR Green PCR Master Mix is a convenient premix for real-time PCR using the SYBR Green I dye. Direct detection of the PCR product is monitored by measuring the increase in fluorescence that is caused by the SYBR Green I dye binding to double-stranded DNA.

About the Reagent Components

AmpliTaq Gold DNA Polymerase

The use of the hot start enzyme AmpliTaq Gold® DNA Polymerase is an integral part of Applied Biosystems Assay Design Guidelines for both TaqMan Probe-based and SYBR Green I dye chemistries. The use of AmpliTaq Gold DNA Polymerase ensures a robust reaction and can dramatically reduce the amount of nonspecific product formation. A further benefit is the simplification of assay setup, which can be performed at room temperature.

MultiScribe Reverse Transcriptase

MultiScribe™ Reverse Transcriptase is a recombinant Moloney Murine Leukemia Virus (MuLV) Reverse Transcriptase.

Using the Universal Thermal Cycling Parameters

All Quantitative Assays designed using Applied Biosystems Assay Design Guidelines can be run using the universal thermal cycling parameters. This eliminates any optimization of the thermal cycling parameters and means that multiple assays can be run on the same plate without sacrificing performance. This benefit is critical when combining two assays into a multiplex 5′ nuclease assay system.

Recommended Thermal Cycling Parameters

DNA/cDNA Quantification

The thermal cycling parameters listed in Table 3-7 are recommended for DNA and cDNA Quantification Assays. The parameters apply to both TaqMan Probe-based and SYBR Green I dye chemistries.

Table 3-7 Thermal cycling parameters for DNA and cDNA quantification assays

Times and Temperatures			
Initial Steps		**PCR (Each of 40 Cycles)**	
AmpErase UNG Activation	**AmpliTaq Gold DNA Polymerase Activation**	**Melt**	**Anneal/Extend**
HOLD	HOLD	CYCLE	
2 min @ 50 °C	10 min @ 95 °C	15 sec @ 95 °C	1 min @ 60 °C

RNA Quantification Using One-Step RT-PCR

The thermal cycling parameters listed in Table 3-8 are recommended for RNA Quantification Assays using one-step RT-PCR. The parameters apply to both TaqMan Probe-based and SYBR Green I dye chemistries.

Table 3-8 Thermal cycling parameters for RNA quantification assays using one-step RT-PCR

Times and Temperatures[a]			
Initial Steps		**PCR (Each of 40 Cycles)**	
Reverse Transcription	**AmpliTaq Gold DNA Polymerase Activation**	**Melt**	**Anneal/Extend**
HOLD	HOLD	40 CYCLES	
30 min @ 48 °C	10 min @ 95 °C	15 sec @ 95 °C	1 min @ 60 °C

a. Not applicable for the TaqMan EZ RT-PCR Kit Protocol. See the TaqMan EZ RT-PCR Kit Protocol for the appropriate values.

RNA Quantification Using Two-Step RT-PCR

The thermal cycling parameters listed in Table 3-9 and Table 3-10 are recommended for RNA Quantification Assays using two-step RT-PCR. The parameters apply to both TaqMan Probe-based and SYBR Green I dye chemistries.

Table 3-9 Thermal cycling parameters for RNA quantification assays using two-step RT-PCR

Times and Temperatures				
1) RT Step	HOLD	HOLD	HOLD	—
	10 min @ 25 °C	30 min @ 48 °C	5 min @ 95 °C	
2) PCR Step	**Initial Steps**		**PCR (Each of 40 Cycles)**	
	AmpErase UNG Activation	**AmpliTaq Gold DNA Polymerase Activation**	**Melt**	**Anneal/ Extend**
	HOLD	HOLD	CYCLE	
	2 min @ 50 °C	10 min @ 95 °C	15 sec @ 95 °C	1 min @ 60 °C

Table 3-10 Thermal cycling parameters for RNA Quantification assays using the high-capacity cDNA archive kit when using two-step RT-PCR

Times and Temperatures				
1) RT Step	HOLD		HOLD	
	Step 1		Step 2	
	10 min @ 25 °C		120 min @ 37 °C	
2) PCR Step	**Initial Steps**		**PCR (Each of 40 Cycles)**	
	AmpErase UNG Activation	**AmpliTaq Gold DNA Polymerase Activation**	**Melt**	**Anneal/ Extend**
	HOLD	HOLD	CYCLE	
	2 min @ 50 °C	10 min @ 95 °C	15 sec @ 95 °C	1 min @ 60 °C

IMPORTANT! For most applications and when large amounts of cDNA are required, Applied Biosystems recommends 120 minutes at 37 °C for reverse transcription to achieve optimal conversion.

Optimizing Primer Concentrations

By independently varying forward and reverse primer concentrations, you can identify the concentrations that provide optimal assay performance. Primers are always in large molar excess during the exponential phase of PCR amplification; by adjusting their initial concentration, their effective melting temperatures can be adjusted.

When using Universal TaqMan PCR Master Mix, Applied Biosystems recommends the primer concentrations shown in Table 3-11. Detailed discussions follow for the:

- Primer optimization matrix (page 3-17)
- 5′ Nuclease quantification assays (using TaqMan Probe-based chemistry, page 3-17)
- SYBR Green I Dye quantification assays (page 3-18)

Default Primer Concentrations

The recommended primer concentrations listed in Table 3-11 are for DNA and cDNA Quantification Assays.

Table 3-11 Recommended primer concentrations for DNA and cDNA quantification assays

Chemistry	Concentrations (nM)	
	Forward Primer	Reverse Primer
TaqMan probe	900	900
SYBR Green I dye	50	50

Primer Optimization Matrix

A primer optimization matrix allows you to determine the minimum primer concentration that will give you the minimum C_T and maximum ΔR_n.

A primer optimization matrix can help to compensate for nonspecific primer binding, which can reduce the amount of primer available to bind at its specific site.

5′ Nuclease Quantification Assays

For a 5′ nuclease quantification assay, optimal performance is achieved by selecting the primer concentrations that provide the lowest C_T and highest ΔR_n for a fixed amount of target template.

The results of a typical TaqMan primer optimization matrix experiment are shown in Figure 3-2. Figure 3-2(a) shows the amplification plots for all primer concentration combinations in linear view. Figure 3-2(b) shows the same data in log view format. The combination of 50-nM forward and reverse primer (Plot group C) gives both the lowest ΔR_n and highest C_T. All other primer combinations that contain a 150-nM concentration of either the forward or reverse primer (Plot group B) give a reduced ΔR_n . All primer combinations that contain at least 300-nM forward and reverse primer (Plot group A) give both the highest ΔR_n and the lowest C_T; as a result, any of the plot group A or B would provide optimal performance.

It should be noted that, although C_T values are the parameter by which quantitative values are assigned in a real-time Quantification Assay, ΔR_n values can also prove important when trying to obtain maximum sensitivity and reproducibility.

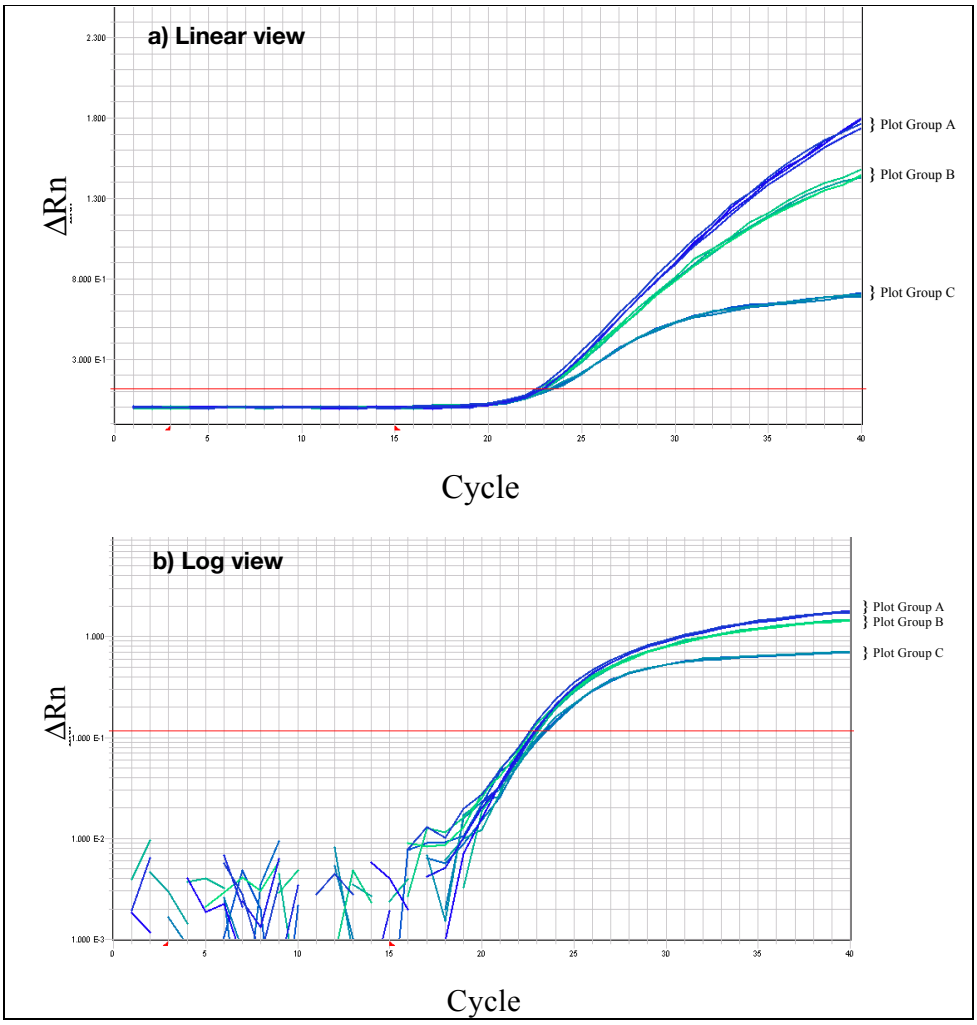

Figure 3-2 Primer optimization experimental results showing amplification plots of primer combinations.
Plot group key:
A: Combinations that contain at least 300 nM of forward and reverse primer
B: Combinations that contain at least 150 nM of forward and reverse primer
C: Combinations that contain at least 50 nM of forward and reverse primer

SYBR Green I Dye Quantification Assays

Optimizing primer concentrations is slightly more complex for a SYBR Green I dye Quantification Assay. The same primer optimization matrix should be performed; however, this time it must include NTCs. In this case, the primer concentrations selected should provide a low C_T and high ΔR_n when run against the target template, but should not produce nonspecific product formation with NTCs. An ideal NTC amplification plot is shown in Figure 3-1 on page 3-6.

Dissociation curves or gel analysis can be extremely useful when selecting optimal primer concentrations for a SYBR Green I dye Quantification Assay. This is demonstrated in Figure 3-3 on page 3-19, which shows the results from a primer optimization matrix at primer concentrations of 900-nM forward and reverse primers. The strong amplification of the NTC wells shown in Figure 3-3(a) indicates that significant nonspecific amplification is occurring. This is confirmed by the dissociation curve data shown in Figure 3-3(b), which shows that the melting

temperature of the product generated in the absence of template is lower than the melting temperature of the specific product generated with template. This is typical of primer-dimer formation and indicates that lower primer concentrations may provide more optimal results.

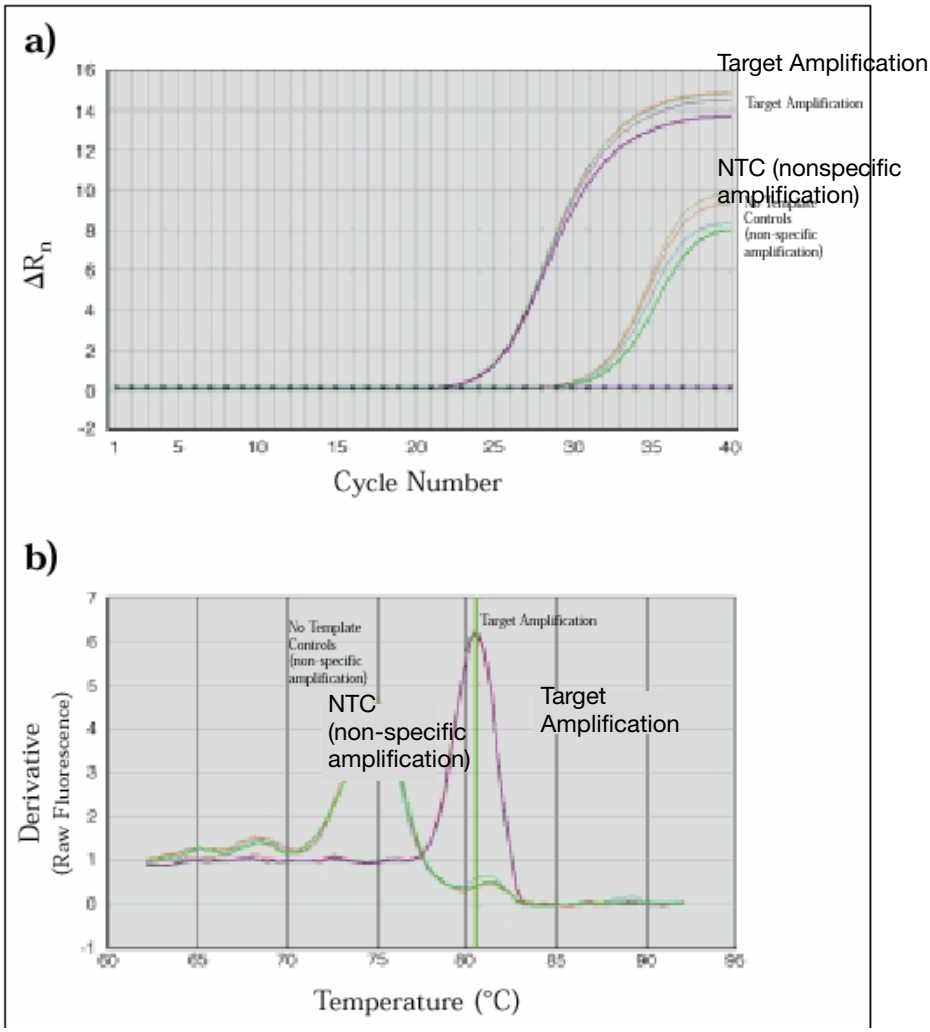

Figure 3-3 Amplification data using SYBR Green I dye chemistry.
(a) Amplification plot (linear view) demonstrating suspected nonspecific amplification in NTC wells.
(b) Dissociation curve analysis confirming product in NTC wells has a different melting temperature from the specific product.

Optimizing the Probe Concentration

The recommended probe concentration of 250 nM ensures excellent assay performance. However, depending on the requirements of the assay, a probe optimization experiment can prove useful.

Note: No probe is required for SYBR Green I dye detection.

Recommended Probe Concentrations

The recommended probe concentrations for DNA and cDNA Quantification Assays using TaqMan Probe-based chemistry is 250 nM.

Figure 3-4 shows the results of a probe optimization experiment in which the probe concentration is varied from 50 to 250 nM. Figure 3-4(a) shows an increase in ΔR_n as the probe concentration is increased, whereas Figure 3-4(b) shows that the C_T value changes for all concentrations of probe.

It should be noted, however, that to ensure the best reproducibility, especially when wishing to detect low copy numbers of a target sequence, it is necessary to avoid probe limiting concentrations. The assay should be run at a probe concentration of 250 nM. By using a 250 nM concentration, probe limitation is avoided and large ΔR_n values are ensured. Large ΔR_n values indicate a robust assay that is performing at high efficiency, giving high product yield and allowing more accurate peak measurement.

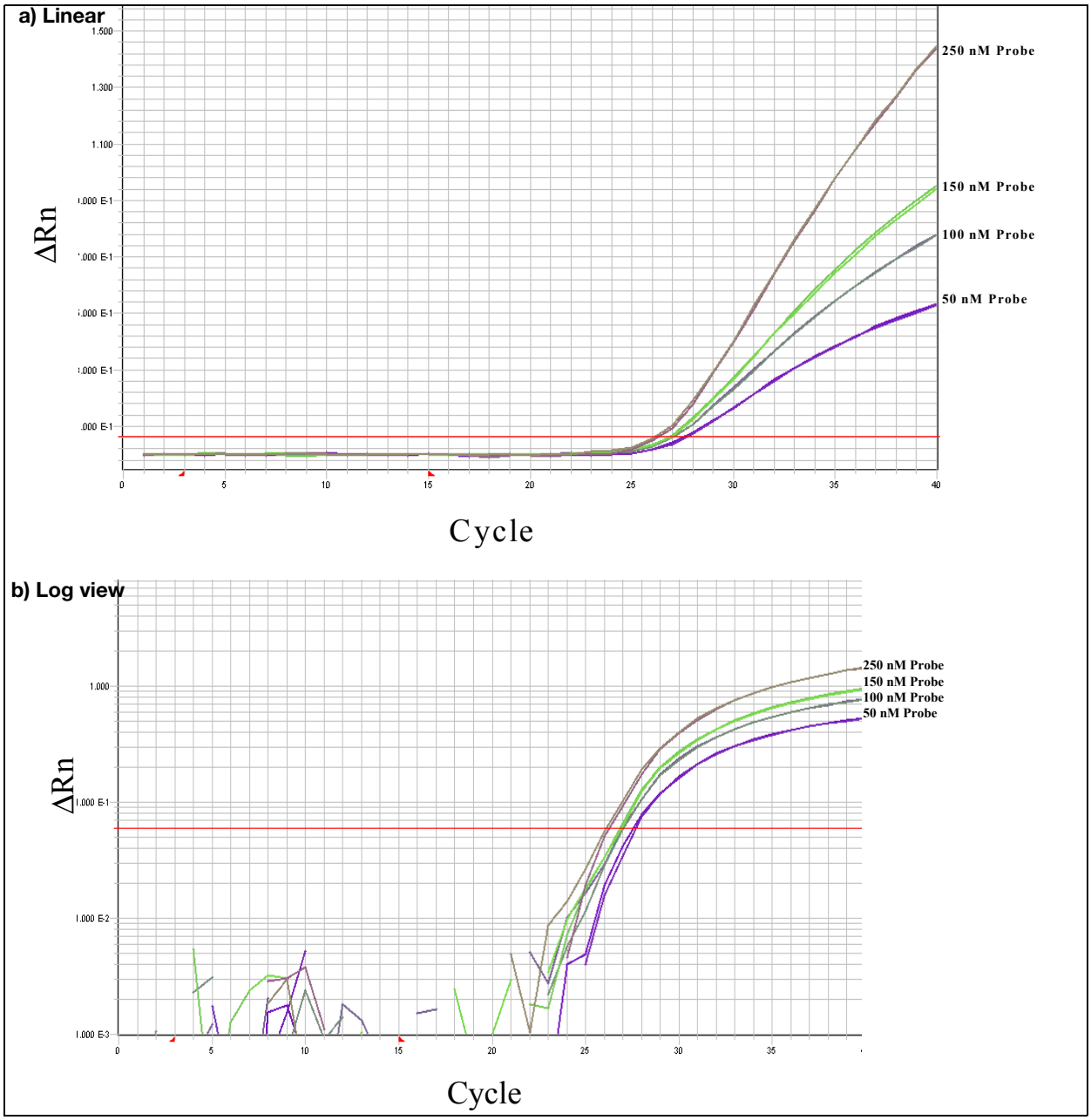

Figure 3-4 Amplification plot (linear and log views) of probe concentration titration from 50 to 250 nM.

Using Multiplex PCR

Multiplex PCR is the use of more than one primer/probe set in the same tube. Multiplex PCR is most commonly used in 5′ nuclease assays for the relative quantification of gene expression.

Typically one probe (labeled with FAM™ dye) is used to detect the target species; another probe (labeled with VIC® dye) is used to detect an endogenous control (internal control gene). Running both assays in a single tube reduces both the running costs and the dependence on accurate pipetting when splitting a sample into two separate tubes.

Multiplex in Contrast to Singleplex

In order to multiplex using the comparative C_T method, you must ensure that the endogenous control that you have selected is more abundant (lower C_T) than all of the targets that you are trying to quantify under all conditions and then you must run the endogenous control assay as a primer-limited assay. For multiplexing, the endogenous control assay (for the more abundant template) in each reaction must be primer limited to avoid competitive PCR that may alter the C_T of the less abundant template.

Primer limited endogenous control reactions are used in multiplex assays to normalize for the amount of input nucleic acid into different wells as well as to normalize for effects on the target assay in the same well. However, a primer-limited assay may be more susceptible to fluctuations in reaction conditions than the primer non-limited target assay that it is normalizing.

Doing this in a multiplex format becomes increasingly more complex as the number of targets you wish to quantify increases. It becomes increasingly unlikely that you will be able to identify a suitable endogenous control that will be more abundant than all of the targets you wish to query and whose expression does not change as a result of the experimental conditions or across different samples.

As a result, for an increasing number of targets, it is likely to be more efficient and effective to run the targets and controls in the singleplex format utilizing the precision of the sequence detection system in conjunction with delivery of equivalent amounts of input material to different reaction wells.

For these reasons, when you analyze multiple numbers of targets it is likely to be more effective to run your assays in the singleplex format. To multiplex, you would have to first run all of your target assays and endogenous control assays in both the multiplex and singleplex format and compare C_T values from both formats to determine if there are any effects of the multiplexing on your C_T values—which could be a larger undertaking than the study itself.

Also by using the singleplex method, any target can potentially be used as an endogenous control at the analysis step of the process. Any target whose expression level does not change with experimental conditions or across samples may serve as an endogenous control. Therefore, the more targets you have in a singleplex format, the higher the probability that you will have one or more suitable endogenous controls against which to normalize your remaining targets.

Primer Limiting in Multiplex Assays

To generate an accurate multiplex assay, it is important to ensure that the amplification of one species does not dominate the other. Otherwise, the amplification of a highly abundant species can prevent the less abundant species from amplifying efficiently. Such a scenario could easily produce inaccurate results and, in severe cases, inhibit detection of the less abundant species completely. This situation can be avoided by limiting the concentrations of the primers used to amplify the more abundant species, thereby "turning off" the amplification soon after the C_T has been established.

Primer limitation results in the reaction components common to both assays not being exhausted, allowing the amplification of the less abundant species to continue at high efficiency. If the more abundant species is not known, it should be determined before entering into a multiplex assay system by running both targets in separate tubes. Both amplifications should be primer limited if neither species is consistently more abundant.

Considering Relative Abundance of the Target and Reference

In applying the primer limitation to target and endogenous control amplifications, the relative abundance of the two species must be considered. For quantification of gene expression, it is possible to use rRNA as an endogenous control. The concentration of rRNA in total RNA is always greater than the concentration of any target mRNA. Therefore, in multiplex reactions amplifying both target and rRNA, only the concentrations of the rRNA primers need to be limited.

Limiting Primer Matrix

To define limiting primer concentrations, run a matrix of forward and reverse primer concentrations using the value of the minimum initial template. The goal is to identify primer concentrations that reduce the ΔR_n of the assay without affecting the C_T value. Table 3-12 illustrates a recommended matrix of forward and reverse primers varying in concentration from 20 to 100 nM.

Note: Although following all design criteria does facilitate the ability to identify limiting primer concentrations, it may not be possible for all assays. If a limiting primer matrix experiment does not enable the identification of primer limiting concentrations, it will be necessary to redesign at least one primer or run the reactions in separate tubes.

Table 3-12 Matrix of varying concentrations of forward and reverse primers (20 to 100 nM)

Forward: Reverse:	100 nM 100 nM	100 nM 80 nM	100 nM 60 nM	100 nM 40 nM	100 nM 20 nM
Forward: Reverse:	80 nM 100 nM	80 nM 80 nM	80 nM 60 nM	80 nM 40 nM	80 nM 20 nM
Forward: Reverse:	60 nM 100 nM	60 nM 80 nM	60 nM 60 nM	60 nM 40 nM	60 nM 20 nM
Forward: Reverse:	40 nM 100 nM	40 nM 80 nM	40 nM 60 nM	40 nM 40 nM	40 nM 20 nM
Forward: Reverse:	20 nM 100 nM	20 nM 80 nM	20 nM 60 nM	20 nM 40 nM	20 nM 20 nM

Example

The results of a limiting primer matrix experiment are shown in Figure 3-5. Figure 3-5(a) shows that only when lowering the primer concentrations below approximately 50 nM is the C_T value significantly affected. Figure 3-5(b) shows the corresponding relationship between primer concentrations and ΔR_n , and demonstrates that lower product yields can be achieved by decreasing forward and reverse primer concentrations.

The plateau area visible in Figure 3-5(a) shows the region in which suitable primer limiting concentrations can be found. In this area, the C_T (and therefore the corresponding quantification value) is unchanged, whereas the ΔR_n value and corresponding product yield are significantly reduced.

For this example, an appropriate selection of primer limiting concentrations would be at least 50 nM forward and reverse primer. It is important to note that probe concentration should be kept at an optimal level even when an assay is primer limited to ensure that the signal produced is large enough for accurate multicomponenting by the Sequence Detection System software.

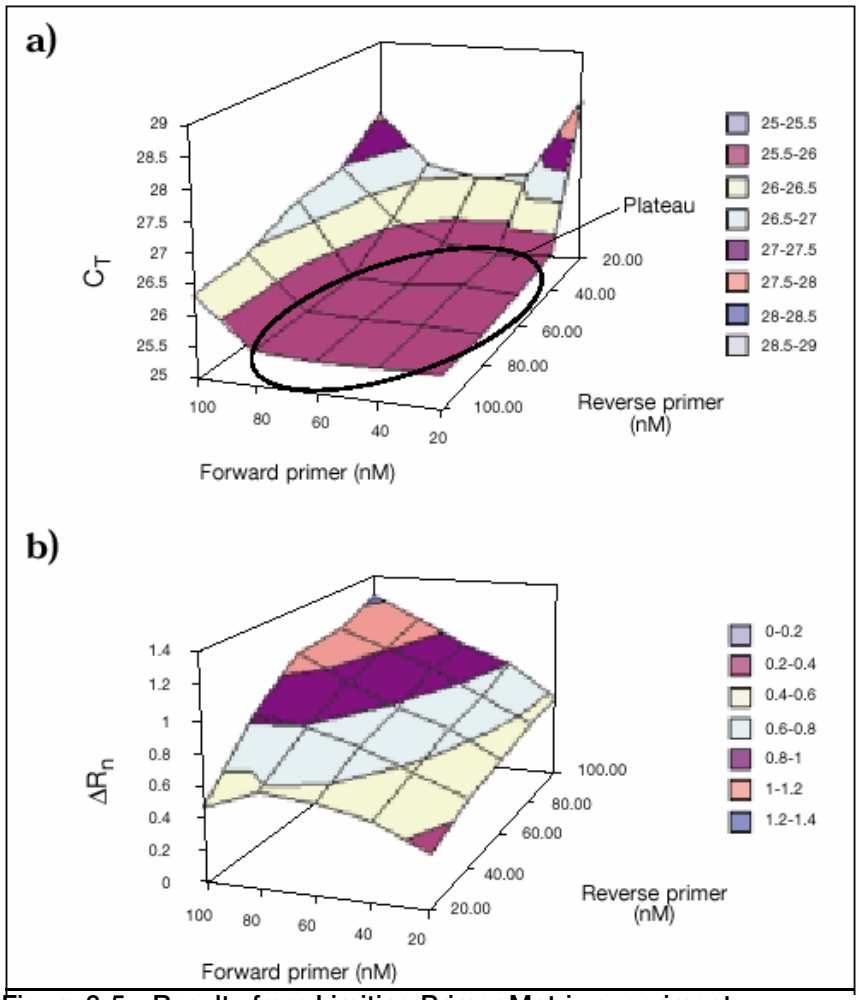

Figure 3-5 Results from Limiting Primer Matrix experiment.
(a) Shows how C_T value is affected by variation in forward and reverse primer concentrations. Plateau region indicated shows area where C_T value remains constant.
(b) Shows reduction in ΔR_n Values as primer concentration are decreased.

Section 3.2 Selecting a Data Analysis Approach and Determining Results

In This Section

The following topics are covered in this section:

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Data Analysis. 3-26
Relative or Absolute Quantification 3-26
Relative Standard Curve Method for Quantification 3-29
Comparative CT Method for Relative Quantification. 3-35
Multiplex PCR (Same-Tube) Method for Relative Quantification 3-40
Standard Curve Method for Absolute Quantification 3-46

Data Analysis

Data analysis varies depending on the product, assay, and instrument. Refer to the appropriate instrument user guide for instructions on how to analyze your data.

General Process

The general process for analyzing the data from gene expression assays involves the following procedures:

1. Viewing the amplification plots for the entire plate
2. Setting the baseline and threshold values
3. Using the methods detailed in this section to determine results

Resources for Data Analysis

For more information about analyzing your data, see Livak and Schmittgen, 2001.

Also refer to the following documents:

- The appropriate instrument user guide
- User Bulletin #2: *Relative Quantification of Gene Expression* (PN 4303859)
- Data Analysis and Relative Quantification chapters in the *TaqMan Cytokine Gene Expression Plate I Protocol* (PN 4306744). This protocol provides examples using multiplex reactions.
- *RQ Manager Software User Guide* (PN 4339753)
- *ABI PRISM® 7900HT Real Time PCR System User Guide* (PN 4317596)
- *SDS Enterprise Database Administration Guide* (PN 4346513)

Note: Some documents are available through the Internet (see "How to Obtain Support" on page xi).

Relative or Absolute Quantification

You can design your assay as relative or absolute quantification. How you calculate the results of your Quantification Assays depends on your experimental design.

What Is Relative Quantification?

Relative quantification describes the change in expression of the target gene in a test sample relative to a calibrator sample. The calibrator sample can be an untreated control or a sample at time zero in a time-course study (Livak and Schmittgen, 2001). Relative quantification provides accurate comparison between the initial level of template in each sample.

Example

You can compare the level of expression of a gene (Gene A) between a treated and untreated sample (for example, a drug treatment) relative to the expression of a second gene (Gene B). The resulting data is the expression of Gene A in treated and untreated samples normalized to the expression of Gene B.

Calculation Methods for Relative Quantification

Relative quantification can be performed with data from all of the SDS instruments. The calculation methods used for relative quantification are:

- Relative standard curve method, singleplex PCR
- Comparative C_T method ($\Delta\Delta C_T$), singleplex PCR
- Relative standard curve method, multiplex PCR
- Comparative C_T method, multiplex PCR

Note: Assays-on-Demand Product and Assays-by-Design Service products are intended for use in singleplex reactions.

Determining Which Method to Use

All these methods can give equivalent results. When determining which method you want to use, consider the following:

- **Relative Standard Curve Method**–Running the target and endogenous control amplifications in separate tubes and using the relative standard curve method of analysis requires the least amount of optimization and validation.
- **Comparative C_T Method**–To use the comparative C_T method, a validation experiment must be run to show that the efficiencies of the target and endogenous control amplifications are approximately equal. The advantage of using the comparative C_T method is that the need for a standard curve is eliminated. This increases throughput because wells no longer need to be used for the standard curve samples. It also eliminates the adverse effect of any dilution errors made in creating the standard curve samples. Here, the target and endogenous control amplifications are run in separate tubes.
- **Multiplex PCR**–To amplify the target and endogenous control in the same tube, limiting primer concentrations must be identified and shown not to affect C_T values. By running the two reactions in the same tube, throughput is increased and the effects of pipetting errors are reduced.

What Is Absolute Quantification?

Absolute quantification determines the input copy number of the transcript of interest, usually by relating the PCR signal to a standard curve (Livak and Schmittgen, 2001).

Example

Use absolute quantification to measure viral copy number in samples for which this information is not known (unknown samples). In order to measure the viral copy number in the unknown samples, compare the measurement to a standard curve of known viral copy numbers. Since the basis for measurement is a standard curve with known quantities of virus, the measurement would be absolute.

Calculation Methods for Absolute Quantification

Absolute quantification can be performed with data from all of the SDS instruments, however, the absolute quantities of the standards must first be measured by some independent means.

The calculation method used for absolute quantification is the standard curve method.

Terms Used

The terms in Table 3-13 are used in this discussion of absolute and relative quantification.

Table 3-13 Terms used in absolute and relative quantification

Control/Term	Definition
Standard	A sample of known concentration used to construct a standard curve.
Reference	A passive or active signal used to normalize experimental results. Endogenous and exogenous controls are examples of active references. Active reference means the signal is generated as the result of PCR amplification. The active reference has its own set of primers and probe. • Endogenous control – This is an RNA or DNA that is present in each experimental sample as isolated. By using an endogenous control as an active reference, you can normalize quantification of a messenger RNA (mRNA) target for differences in the amount of total RNA added to each reaction. • Exogenous control – This is a characterized RNA or DNA spiked into each sample at a known concentration. An exogenous active reference is usually an *in vitro* construct that can be used as an internal positive control (IPC) to distinguish true target negatives from PCR inhibition. An exogenous reference can also be used to normalize for differences in efficiency of sample extraction or complementary DNA (cDNA) synthesis by reverse transcriptase.
Passive reference	A dye that provides an internal reference to which the reporter dye signal is normalized. Whether or not an active reference is used, it is important to use a passive reference (for example, ROX dye) in order to normalize for non-PCR-related fluctuations in fluorescence signal.
Normalized amount of target	A unitless number that can be used to compare the relative amount of target in different samples.
CV	Coefficient of variation. The ratio of the standard deviation of a distribution to its arithmetic mean.
Calibrator	In relative quantification, the sample used as the basis for comparative results.
Endogenous control	Internal control gene present in each experimental sample. By using an endogenous control as an active reference, you can normalize quantification of a messenger RNA (mRNA) target for differences in the amount of total RNA added to each reaction.

For More Information

For more information on absolute and relative quantification, refer to Livak and Schmittgen, 2001. This document contains detailed procedures for performing the experiments referenced in this section.

Relative Standard Curve Method for Quantification

It is easy to prepare standard curves for relative quantification because quantity is expressed relative to some basis sample, such as the calibrator.

For all experimental samples, target quantity is determined from the standard curve and divided by the target quantity of the calibrator. Thus, the calibrator becomes the 1× sample, and all other quantities are expressed as an *n*-fold difference relative to the calibrator. For example, in a study of drug effects on expression, the untreated control would be an appropriate calibrator.

Requirements

The requirements below are critical for proper use of the relative standard curve method for quantification:

- It is important that stock RNA or DNA be accurately diluted, but the units used to express this dilution are irrelevant. If two-fold dilutions of a total RNA preparation from a control cell line are used to construct a standard curve, the units could be the dilution values 1, 0.5, 0.25, 0.125, and so on. By using the same stock RNA or DNA to prepare standard curves for multiple plates, the relative quantities determined can be compared across the plates.
- For quantification normalized to an endogenous control, standard curves are prepared for both the target and the endogenous control. For each experimental sample, the amount of target and endogenous control is determined from the appropriate standard curve. Then, the target amount is divided by the endogenous control amount to obtain a normalized target value.
- Again, one of the experimental samples is the calibrator, or 1× sample. Each of the normalized target values is divided by the calibrator normalized target value to generate the relative expression levels.
- Pipette correctly and accurately. Pipette volumes greater than 5 µl of nucleic acid sample to minimize inaccuracy.

Endogenous Control

Amplification of an endogenous control can be performed to standardize the amount of sample RNA or DNA added to a reaction. For the quantification of gene expression, researchers have used ß-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA (rRNA), or other RNAs as an endogenous control.

When using the comparative C_T method with real-time PCR, you can normalize to an endogenous reference using data generated during the PCR experiment. This is especially useful when you have a limited amount of RNA or when performing high-throughput processing of a large number of samples (Livak and Schmittgen, 2001).

Another approach is to normalize to a measurement external to the PCR experiment. For example, you can use UV absorption to determine the amount of RNA added to a cDNA reaction. You then run a PCR using cDNA derived from the same amount of input RNA. You can use this approach to select an appropriate endogenous control and to determine if an endogenous control is affected by the treatment. In this case, the target gene and the endogenous reference are the same item (Livak and Schmittgen, 2001).

Standards

As the sample quantity is divided by the calibrator quantity, the unit from the standard curve drops out. Thus, all that is required of the standards is that their relative dilutions be known. For relative quantification, this means any stock RNA or DNA containing the appropriate target can be used to prepare standards.

How to Perform the Relative Standard Curve Method

To perform the relative standard curve method for quantification:

- Perform a run on your SDS instrument, this includes:
  - Setting up a reaction plate
  - Analyzing the data
  - Creating a standard curve
- Determine the relative values.

See "Example of the Relative Standard Curve Method" below for an illustration of these steps.

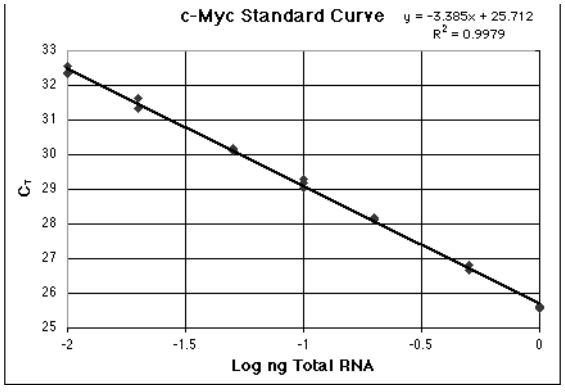
Example of the Relative Standard Curve Method

This example illustrates the use of standard curves for relative quantification, based on:

- The target is human c-myc mRNA, and the endogenous control is human GAPDH mRNA.
- The target and endogenous control are amplified in separate tubes.
- Dilutions of a cDNA sample prepared from total Raji RNA are used to construct standard curves for the c-myc and the GAPDH amplifications.
- The unknown samples (samples to characterize) are cDNA prepared from total RNA isolated from human brain, kidney, liver, and lung.

Performing the Run

The procedure below is a general outline for performing a run. Refer to your instrument's user manual for detailed instructions.

1.	Set up a reaction plate.
2.	Place the reaction plate on your SDS instrument and start the run.
3.	When the run is complete, analyze the data.
4.	Set the threshold and create a standard curve from the data. The figure below shows the standard curve for the amplification of the c-myc target detected using a FAM dye labeled probe. 

Determining the Relative Values

To determine the relative value:

1.	Calculate the log input amount by entering the following formula in one cell of the work sheet of any spreadsheet program: = ([cell containing C_T value] – b)/m where b = y-intercept of standard curve line and m = slope of standard curve line **Note:** In this example, b = 25.712 and m = -3.385 for the equation y = mx + b.
2.	Calculate the input amount by entering the following formula in an adjacent cell: = 10^ [cell containing log input amount] **Note:** The units of the calculated amount are the same as the units used to construct the standard curve, which are nanograms of Total Raji RNA. If it is calculated that an unknown has 0.23 ng of Total Raji RNA, then the sample contains the same amount of c-myc mRNA found in 0.23 ng of the Raji Control RNA.
3.	Repeat the steps to construct a standard curve for the endogenous reference using the C_T values determined with the GAPDH probe. Refer to Table 3-14 on page 3-33.
4.	Because c-myc and GAPDH are amplified in separate tubes, average the c-myc and GAPDH values separately.
5.	Divide the amount of c-myc by the amount of GAPDH to determine the normalized amount of c-myc (c-myc_N).
6.	Designate the calibrator. In Table 3-14 on page 3-33, brain is arbitrarily designated as the calibrator.
7.	Divide the averaged sample (kidney, liver, or lung) value by the averaged calibrator (brain) value. Calculate the coefficient of variation (see below), based on the cv of the sample and brain.

Calculating the Coefficient Of Variation

The c-myc$_N$ value is determined by dividing the average c-myc value by the average GAPDH value. The standard deviation of the quotient is calculated from the standard deviations of the c-myc and GAPDH values using the following formula:

$$cv = \sqrt{cv_1^2 + cv_2^2}$$

where:

$$cv = \frac{s}{\overline{X}} = \frac{stddev}{meanvalue}$$

As an example, from Table 3-14 on page 3-33 (brain sample):

$$cv_1 = \frac{0.004}{0.039}$$

and

$$cv_2 = \frac{0.034}{0.54}$$

$$cv = \sqrt{\left(\frac{0.004}{0.039}\right)^2 + \left(\frac{0.034}{0.54}\right)^2} = 0.12$$

since

$$cv = \frac{s}{\overline{X}}$$

$$s = (cv)(\overline{X})$$

$$s = (0.12)(0.07)$$

$$s = 0.008$$

Comparing Samples with a Calibrator

The normalized amount of target (c-myc$_N$) is a unitless number that can be used to compare the relative amount of target in different samples. One way to make this comparison is to designate one of the samples as a calibrator. In Table 3-14 on page 3-33, brain is designated as the calibrator; brain is arbitrarily chosen because it has the lowest expression level of the target.

Relative Standard Curve Method Results

Each c-myc$_N$ value in Table 3-14 on page 3-33 is divided by the brain c-myc$_N$ value to give the values in the final column. These results indicate the kidney sample contains 5.5 times as much c-myc mRNA as the brain sample, liver 34.2 times as much, and lung 15.7 times as much.

Note: Averages and standard deviations are calculated from unrounded data, not the rounded data presented here.

Table 3-14 Amounts of c-myc and GAPDH in human brain, kidney, liver, and lung tissues

Tissue	c-myc ng Total Raji RNA	GAPDH ng Total Raji RNA	c-myc$_N$ Norm. to GAPDH[a]	c-myc$_N$ Rel. to Brain[b]
Brain (Designated calibrator)	0.033	0.51		
	0.043	0.56		
	0.036	0.59		
	0.043	0.53		
	0.039	0.51		
	0.040	0.52		
Average	0.039±0.004	0.54±0.034	0.07±0.008	1.0±0.12
Kidney	0.40	0.96		
	0.41	1.06		
	0.41	1.05		
	0.39	1.07		
	0.42	1.06		
	0.43	0.96		
Average	0.41±0.016	1.02±0.052	0.40±0.025	5.5±0.35
Liver	0.67	0.29		
	0.66	0.28		
	0.70	0.28		
	0.76	0.29		
	0.70	0.26		
	0.68	0.27		
Average	0.70±0.036	0.28±0.013	2.49±0.173	34.2±2.37

Table 3-14 Amounts of c-myc and GAPDH in human brain, kidney, liver, and lung tissues *(continued)*

Tissue	c-myc ng Total Raji RNA	GAPDH ng Total Raji RNA	$c\text{-}myc_N$ Norm. to GAPDH[a]	$c\text{-}myc_N$ Rel. to Brain[b]
Lung	0.97	0.82		
	0.92	0.88		
	0.86	0.78		
	0.89	0.77		
	0.94	0.79		
	0.97	0.80		
Average	0.93±0.044	0.81±0.041	1.15±0.079	15.7±1.09

a. The $c\text{-}myc_N$ value is determined by dividing the average c-myc value by the average GADPH value. The standard deviation of the quotient is calculated from the standard deviations of the c-myc and GADPH values.

b. The calculation of $c\text{-}myc_N$ relative to brain involves division by the calibrator value. This is a division by an arbitrary constant, so the cv of this result is the same as the cv for $c\text{-}myc_N$.

Comparative C_T Method for Relative Quantification

The comparative C_T method is similar to the relative standard curve method, except it uses an arithmetic formula rather than a standard curve to achieve the same result for relative quantification.

IMPORTANT! It is possible to eliminate the use of standard curves for relative quantification as long as a validation experiment is performed. (For more information on performing a validation experiment, see below.)

Formula

The amount of target, normalized to an endogenous control and relative to a calibrator, is given by:

$$2^{-\Delta\Delta C_T}$$

See Appendix A, "Formulas," for a derivation of the formula.

Performing the Validation Experiment

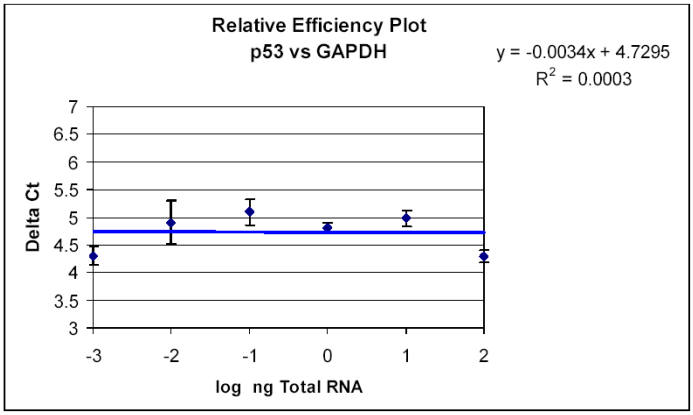
Before using the $\Delta\Delta C_T$ method for quantification, perform a validation experiment like the one below to demonstrate that efficiencies of target and reference are approximately equal.

To perform the validation experiment:

1.	Choose your target and endogenous control.
2.	Perform a dilution series of different input amounts for your target and endogenous control. **Note:** When possible, serial dilutions should cover 5 to 6 logs.
3.	Run the samples on your SDS instrument.
4.	Analyze your data.
5.	Calculate the average C_T and ΔC_T values for your target and endogenous control (see Table 3-15).

To perform the validation experiment: *(continued)*

6. Plot the log of the input amount vs. ΔC_T (see the figure below).



As a guideline, the absolute value of the slope of log input amount vs. ΔC_T should less than 0.1.

The the slope in the above figure is –0.0034, which passes this test.

Note: The less than 0.1 guideline should provide gene expression results with a low degree of experimental variation. However, if it is essential to quantify any variation introduced by the comparative C_T method, parallel experiments using the comparative C_T method and the standard curve method should be performed and the results compared.

If the efficiencies of the two systems are...	Then...
< 0.1	• You can use the $\Delta\Delta C_T$ calculation for the relative quantification of target without running standard curves on the same plate.
> 0.1	• Perform the validation experiment over a larger dynamic range (5 to 6 logs) or design and synthesize new primers to improve efficiency. • Perform the quantification using the standard curve method.

Table 3-15 Average C_T value for c-myc and GAPDH at different input amounts

Input Amount ng Total RNA	c-myc Average C_T	GAPDH Average C_T	ΔC_T c-myc – GAPDH
100.0	20.28 ± 0.11	15.23 ± 0.02	5.05 ± 0.11
10.0	23.88 ± 0.14	18.62 ± 0.04	5.26 ± 0.15
1.0	27.33 ± 0.09	22.18 ± 0.02	5.15 ± 0.09
0.1	30.93 ± 0.23	25.53 ± 0.07	5.40 ± 0.24
0.01	34.39 ± 0.37	29.13 ± 0.14	5.26 ± 0.39
0.001	37.03 ± 0.12	32.57 ± 0.13	4.46 ± 0.17

Relative Efficiency of Target and Reference

For the $\Delta\Delta C_T$ calculation to be valid, the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal. A sensitive method for assessing if two amplicons have the same efficiency is to look at how ΔC_T varies with template dilution. The standard curves for c-myc and GAPDH used in the previous section provide the necessary data. Table 3-15 shows the average C_T value for c-myc and GAPDH at different input amounts.

How to Perform the Comparative C_T Method

To perform the comparative C_T method for relative quantification:

- Perform a run on your SDS instrument. This includes:
  - Setting up a reaction plate
  - Analyzing the data
- Determine the ΔC_T value (Target –Endogenous control).
- Perform the $\Delta\Delta C_T$ calculation to determine fold difference in gene expression (ΔC_T Target –ΔC_T Calibrator).

See the example on below for an illustration of these steps.

Example of the Comparative C_T Method

This example illustrates the use of the comparative C_T method for relative quantification. In this example:

- The target is human c-myc mRNA and the endogenous control is human GAPDH mRNA.
- The target and endogenous control are amplified in separate tubes.
- The unknown samples are cDNA prepared from total RNA isolated from human brain, kidney, liver, and lung.

Performing the Run

The procedure below is a general outline for performing a run. Refer to your instrument user manual for detailed instructions.

To perform the run:

1.	Set up a reaction plate.
2.	Place the reaction plate on your instrument and start the run.
3.	When the run is complete, analyze the data.

Determining the ΔC_T Value and Standard Deviation

The ΔC_T value is determined by subtracting the average GAPDH C_T value from the average c-myc C_T value. The standard deviation of the difference is calculated from the standard deviations of the c-myc and GAPDH values using the following formula:

$$s = \sqrt{s_1^2 + s_2^2}$$

where: s = std dev

As an example, from Table 3-16 on page 3-39 (brain sample):

$$s_1 = 0.15$$

and

$$s_2 = 0.09$$

$$s = \sqrt{(0.15)^2 + (0.09)^2} = 0.17$$

Performing the $\Delta\Delta C_T$ Calculation

To perform the $\Delta\Delta C_T$ calculation:

1.	Determine the ΔC_T by subtracting the average C_T of your endogenous control from the average C_T of your target.
2.	Determine the $\Delta\Delta C_T$ by subtracting the ΔC_T of your calibrator from the ΔC_T of your test sample or treated sample.

Comparative C_T Method Results

The C_T data used to determine the amounts of c-myc and GAPDH mRNA shown in Table 3-15 on page 3-37 are used to illustrate the $\Delta\Delta C_T$ calculation. Table 3-16 below shows the average C_T results for the human brain, kidney, liver, and lung samples and how these C_T s are manipulated to determine ΔC_T , $\Delta\Delta C_T$, and the relative amount of c-myc mRNA. The results are comparable to the relative c-myc levels determined using the standard curve method.

Although the comparative C_T method can be used to make this type of tissue comparison, biological interpretation of the results is complex. The single relative quantity reported actually reflects variation in both target and reference transcripts across a variety of cell types that might be present in any particular tissue (Livak and Schmittgen, 2001).

Table 3-16 Relative quantification using the comparative C_T method

Tissue	Target c-myc Average C_T	Endogenous Control GAPDH Average C_T	ΔC_T c-myc–GAPDH[a]	$-\Delta\Delta C_T$ $-(\Delta C_T - \Delta C_{T,\ Brain})$[b]	c-myc$_N$ Rel. to Brain[c]
Brain Calibrator	30.49±0.15	23.63±0.09	6.86±0.17	0.00±0.17	1.0 (0.9 to 1.1)
Kidney	27.03±0.06	22.66±0.08	4.37±0.10	2.50±0.10	5.6 (5.3 to 6.0)
Liver	26.25±0.07	24.60±0.07	1.65±0.10	5.21±0.10	37.0 (34.5 to 39.7)
Lung	25.83±0.07	23.01±0.07	2.81±0.10	4.05±0.10	16.5 (15.4 to 17.7)

a. The ΔC_T value is determined by subtracting the average GAPDH C_T value from the average c-myc C_T value.
For example, ΔC_T Brain = 30.49 - 23.63 = 6.86.
The standard deviation of the difference is calculated from the standard deviations of the c-myc and GAPDH values.

b. The calculation of $-\Delta\Delta C_T$ involves subtracting ΔC_T calibrator value from the ΔC_T target value.
For example, $-\Delta\Delta C_T$ Kidney = -(ΔC_T Kidney -ΔC_T Brain) = -(4.37 - 6.86) = 2.50 (using full data value, not the rounded data presented in this table)
This is subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of the ΔC_T value.

c. The range given for c-myc$_N$ relative to brain is determined by evaluating the expression: $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T$ + s and $\Delta\Delta C_T$ – s, where s = the standard deviation of the $\Delta\Delta C_T$ value.
For example, Range Kidney has a $\Delta\Delta C_T$ value of -2.5 and a range of Standard deviation = 0.10. Therefore: $2^{-\Delta\Delta C_T} = 2^{2.50}$ = 5.6. The lower range limit = $2^{-2.50+0.1}$ = 5.3. The upper range limit = $2^{-2.50-0.1}$ = 6.0.

Multiplex PCR (Same-Tube) Method for Relative Quantification

Multiplex PCR is the use of more than one primer pair and probe in the same tube. You can use this method in relative quantification where one primer pair/probe amplifies the target and another primer pair/probe amplifies the endogenous control in the same tube.

You can perform a multiplex reaction for both the standard curve method and the comparative C_T method.

Advantages of Multiplex PCR

The advantages of performing target and reference reactions in the same tube are:

- Higher throughput is most evident if you are interested in analyzing a single target because the number of sample tubes is reduced by a factor of two.
- More efficient use of samples
- Reduction in reagent use and cost

See “Primer Limiting in Multiplex Assays” on page 3-23.

About Multiple Reporter Dyes

The TaqMan Probe-based chemistry includes multiple reporter dyes, which make it possible to amplify and detect target amplicon and endogenous control amplicon in the same tube.

The reporter dyes recommended for TaqMan probes are FAM and VIC. These dyes are distinguishable from one another because they have different emission wavelength maxima:

- FAM: λ_{max} = 518 nm
- VIC: λ_{max} = 554 nm

About Multi-componenting

The software for all of the SDS instruments uses a process called multicomponenting to distinguish reporter dyes, the quencher dye TAMRA™ (λ_{max} = 582 nm), if used, and the passive reference ROX™ dye (λ_{max} = 610 nm).

Multicomponenting is a mathematical algorithm that uses pure dye reference spectra to calculate the contribution of each dye to a complex experimental spectrum. When using TaqMan MGB probes, no quencher dye (TAMRA) is necessary. Because there is one less dye to resolve, spectral resolution is improved.

Obtaining Accurate Quantification

For the most accurate quantification using two probes in one tube, use the reporter dyes that have the largest difference in emission maximum: FAM and VIC dye.

About Primer Limitation

Reactions designed to amplify two different sequences in the same tube share common reagents. If the two sequences have different initial copy numbers, it is possible for the more abundant species to use up these common reagents, impairing amplification of the rarer species. For accurate quantification, it is important that the two reactions do not compete. Competition can be avoided by limiting the concentration of primers used in the amplification reactions. For more information on primer limitations, see page 3-23.

How to Perform Multiplex PCR with the Relative Standard Curve Method

To perform the multiplex PCR with the relative standard curve method for quantification:

- Perform a run on your SDS instrument. This includes:
  - Setting up a reaction plate
  - Analyzing the data
  - Creating a standard curve
- Determine the relative values.

See "Example of Multiplex PCR with the Relative Standard Curve Method" below for an illustration of these steps.

Example of Multiplex PCR with the Relative Standard Curve Method

This example illustrates the use of multiplex PCR with the relative standard curve method for quantification. In this example:

- The target is human c-myc mRNA and the endogenous control is human GAPDH mRNA.
- The target and endogenous control are amplified in the same tube.
- Dilutions of a cDNA sample prepared from Total Raji RNA are used to construct standard curves for the c-myc and the GAPDH amplifications.
- The unknown samples are cDNA prepared from total RNA isolated from human brain, kidney, liver, and lung.

Performing the Run

The same-tube and separate tube procedures for the relative standard curve method are identical, with the exceptions noted below.

To perform the run:

1.	Perform the run per the procedures on page 3-30. **Exception:** When you set up the reaction plate, the target amplicon and endogenous control amplicon are in the same tube. **Exception:** ΔC_T is calculated for each reaction and then the ΔC_Ts are averaged.
2.	Determine the relative values per the procedures in Table 3-13 on page 3-31.

Results of the Above Run

Table 3-17 shows the results of a same-tube experiment using the relative standard curve method. In this experiment, the target c-myc normalized to the endogenous control GAPDH was quantified.

Averages and deviations are calculated from unrounded data, not the rounded data presented here.

Table 3-17 Relative quantification using multiplex PCR with the relative standard curve method

Tissue	c-myc ng Total Raji RNA	GAPDH ng Total Raji RNA	$c\text{-}myc_N$ Norm. to GAPDH	$c\text{-}myc_N$ Rel. to Brain
Brain	0.031	0.618	0.05	
	0.038	0.532	0.07	
	0.032	0.521	0.06	
	0.038	0.550	0.07	
	0.032	0.577	0.06	
	0.037	0.532	0.07	
Average			0.06±0.008	1.0±0.14
Kidney	0.365	0.049	0.35	
	0.338	1.035	0.33	
	0.423	1.042	0.41	
	0.334	1.086	0.31	
	0.334	1.021	0.33	
	0.372	1.139	0.33	
Average			0.34±0.035	5.4±0.55
Liver	0.477	0.255	1.87	
	0.471	0.228	2.06	
	0.535	0.258	2.07	
	0.589	0.241	2.44	
	0.539	0.264	2.04	
	0.465	0.227	2.05	
Average			2.09±0.186	33.3±2.97
Lung	0.853	0.085	0.97	
	0.900	0.084	0.88	
	0.956	0.082	1.00	
	0.900	0.093	0.87	
	0.996	0.112	0.87	
	0.859	0.090	0.84	
Average			0.90±0.062	14.4±0.99

How to Perform Multiplex PCR with the Comparative C_T Method

To perform multiplex PCR with the comparative C_T method for relative quantification:

- Perform a validation experiment.
- Perform a run on your SDS instrument. This includes:
  - Setting up a reaction plate
  - Analyzing the data
- Determine the ΔC_T value.
- Perform the $\Delta\Delta C_T$ calculation.

See the example below for an illustration of these steps.

Example of Multiplex PCR with the Comparative C_T Method

This example illustrates the use of multiplex PCR with the comparative C_T method for relative quantification. In this example:

- The target is human c-myc mRNA and the endogenous control is human GAPDH mRNA.
- The target and endogenous control are amplified in the same tubes.
- The unknown samples are cDNA prepared from total RNA isolated from human brain, kidney, liver, and lung.

Procedures

The same-tube and separate tube procedures for the comparative C_T method are identical, with the exceptions noted below.

To perform the run:

1.	Perform the validation experiment per the procedures on page 3-35.
2.	Perform the run per the procedures on page 3-38. **Exception:** When you set up the reaction plate, the target amplicon and endogenous control amplicon will be in the same tube. **Exception:** Because c-myc and GAPDH data are being obtained from the same tube, calculations are carried out individually for each well before averaging.
3.	Determine the ΔC_T value per the procedures on page 3-38.
4.	Perform the $\Delta\Delta C_T$ calculation per the procedures on page 3-38.

Results of the Run

Table 3-18 shows the $\Delta\Delta C_T$ calculations for the same-tube experiment..

Table 3-18 Relative quantification using multiplex PCR with the comparative C_T method

Tissue	c-myc C_T	GAPDH C_T	ΔC_T c-myc - GAPDH	$\Delta\Delta C_T$ ΔC_T - Avg. ΔC_T, Brain	c-myc$_N$ Rel. to Brain
Brain	32.38	25.07	7.31		
	32.08	25.29	6.79		
	32.35	25.32	7.03		
	32.08	25.24	6.84		
	32.34	25.17	7.17		
	32.13	25.29	6.84		
Average			6.93±0.16	0.00±0.16	1.0 (0.9 to 1.1)
Kidney	28.73	24.30	4.43		
	28.84	24.32	4.52		
	28.51	24.31	4.20		
	28.86	24.25	4.61		
	28.86	24.34	4.52		
	28.70	24.18	4.52		
Average			4.47±0.14	–2.47±0.14	5.5 (5.0 to 6.1)
Liver	28.33	26.36	1.97		
	28.35	26.52	1.83		
	28.16	26.34	1.82		
	28.02	26.44	1.58		
	28.15	26.31	1.84		
	28.37	26.53	1.84		
Average			1.81±0.13	–5.12±0.13	34.8 (31.9 to 38.0)

Table 3-18 Relative quantification using multiplex PCR with the comparative C_T method *(continued)*

Tissue	c-myc C_T	GAPDH C_T	ΔC_T c-myc - GAPDH	$\Delta\Delta C_T$ ΔC_T - Avg. ΔC_T, Brain	c-myc$_N$ Rel. to Brain
Lung	27.47	24.55	2.92		
	27.39	24.33	3.06		
	27.30	24.43	2.87		
	27.39	24.32	3.07		
	27.24	24.18	3.06		
	27.46	24.34	3.12		
Average			3.02±0.10	–3.92±0.10	15.1 (14.1 to 16.2)

Standard Curve Method for Absolute Quantification

The standard curve method for absolute quantification is similar to the relative standard curve method, except the absolute quantities of the standards must first be known by some independent means.

Requirements

The guidelines below are critical for proper use of the absolute standard curve method for quantification:

- It is important that the DNA or RNA be a single, pure species. For example, plasmid DNA prepared from *E. coli* often is contaminated with RNA, which increases the A_{260} measurement and inflates the copy number determined for the plasmid.
- Accurate pipetting is required because the standards must be diluted over several orders of magnitude. Plasmid DNA or *in vitro* transcribed RNA must be concentrated in order to measure an accurate A_{260} value. This concentrated DNA or RNA must then be diluted 10^6 to 10^{12} -fold to be at a concentration similar to the target in biological samples.
- The stability of the diluted standards must be considered, especially for RNA. Divide diluted standards into small aliquots, store at –80 °C, and thaw only once before use. An example of the effort required to generate trustworthy standards is provided by Collins (Collins *et al.* 1995), who report on the steps they used in developing an absolute RNA standard for viral RNA quantification.
- It is generally not possible to use DNA as a standard for absolute quantification of RNA because there is no control for the efficiency of the reverse transcription step.

Standards

The absolute quantities of the standards must first be known by some independent means.

Plasmid DNA and *in vitro* transcribed RNA are commonly used to prepare absolute standards. Concentration is measured by A_{260} and converted to the number of copies using the molecular weight of the DNA or RNA.

How to Perform the Absolute Standard Curve Method

Except for preparation of the standards (see above), the absolute standard curve methods and relative standard curve quantification methods are identical.

To perform the absolute standard curve method for quantification, see:

- “How to Perform the Relative Standard Curve Method” on page 3-30
- “Example of the Relative Standard Curve Method” on page 3-30

Allelic Discrimination Assays 4

This chapter covers:

About Allelic Discrimination Assays 4-2
Purchasing an Applied Biosystems Preformulated or Custom-Designed Allelic Discrimination Assay 4-4
Designing Your Own Allelic Assay. 4-6

About Allelic Discrimination Assays

What Is an Allelic Discrimination Assay?

An Allelic Discrimination Assay is an endpoint assay used to determine the genotype of samples. With this assay type, it is possible to differentiate a single nucleotide polymorphism (SNP).

The aim of an Allelic Discrimination Assay is to classify unknown samples as:

- Homozygotes (samples having only allele 1)
- Homozygotes (samples having only allele 2)
- Heterozygotes (samples having both allele 1 and allele 2)

Instruments

Allelic discrimination assays can be used with the following Sequence Detection System (SDS) instruments:

- Applied Biosystems 7900HT Real Time PCR System (7900HT System)
- Applied Biosystems 7300 Real Time PCR System (7300 System)
- Applied Biosystems 7500 Real Time PCR System (7500 System)
- ABI PRISM® 7000 Sequence Detection System (7000 System)
- ABI PRISM® 7700 Sequence Detection System (7700 System)

Note: Use of the 7900HT system, 7000 system, or 7700 system allows for real-time analysis of PCR, which is helpful for troubleshooting. If using a sequence detection system for PCR amplification, perform the endpoint plate read separately.

Note: The GeneAmp® 5700 Sequence Detection System (5700 System) cannot be used for allelic discrimination assays.

Chemistry

Allelic Discrimination Assays can be used with fluorogenic 5′ nuclease chemistry (also known as TaqMan® Probe-based chemistry. For information, see “TaqMan Probe-Based Chemistry” on page 2-3).

Note: The SYBR® Green I dye chemistry is not supported for Allelic Discrimination Assays.

Terms Used in Allelic Discrimination Analysis

Terms commonly used in allelic discrimination analysis are defined in Table 4-1.

Table 4-1 Terms used in allelic discrimination analysis

Term	Definition
No template control (NTC)	A sample that does not contain template. The NTC shows background signal and is used as the negative control. Provides a means of measuring contamination that might give a false positive signal.
Nucleic acid target (also called “target template”)	Nucleotide sequence that you want to detect.

Table 4-1 Terms used in allelic discrimination analysis *(continued)*

Term	Definition
Passive reference	A dye that provides an internal reference to which the reporter dye signal is normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations caused by changes in concentration or volume. A passive reference dye is included in all SDS PCR reagent kits.
Reporter dye	The dye attached to the 5′ end of a TaqMan® probe.
Normalized reporter (R_n)	The normalized intensity of the reporter dye. R_n is calculated by dividing the intensity of the reporter dye by the intensity of the passive reference.
Unknown	The sample you want to classify as either homozygote or heterozygote.

How Allelic Discrimination Assays Work

In allelic discrimination assays, the PCR includes a specific, fluorescent, dye-labeled probe for each allele. You can use TAMRA™ or TaqMan® MGB labelled probes. The probes contain different fluorescent reporter dyes (FAM™ dye and VIC® dye) to differentiate the amplification of each allele.

Each TaqMan MGB probe contains:

- A reporter dye at the 5′ end of each probe
  - VIC dye is linked to the 5′ end of the Allele 1 probe
  - FAM dye is linked to the 5′ end of the Allele 2 probe
- A minor groove-binder (MGB)

  This modification increases the melting temperature (T_m) without increasing probe length (Afonina *et al.*, 1997; Kutyavin *et al.*, 1997), which allows the design of shorter probes. This results in greater differences in T_m values between matched and mismatched probes, which produces more accurate allelic discrimination.
- A nonfluorescent quencher (NFQ) at the 3′ end of the probe

  Because the quencher does not fluoresce, Applied Biosystems sequence detection systems can measure reporter dye contributions more accurately.

During PCR, each probe anneals specifically to complementary sequences between the forward and reverse primer sites. AmpliTaq Gold® DNA polymerase can cleave only probes that hybridize to the allele sequence. Cleavage separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter dye. Thus, the fluorescence signal(s) generated by PCR amplification indicate(s) the alleles that are present in the sample.

Mismatches Between Probe and Allele Sequences

Mismatches between a probe and allele reduce the efficiency of probe hybridization. Furthermore, AmpliTaq Gold DNA polymerase is more likely to displace the mismatched probe rather than cleave it to release reporter dye.

The figure below illustrates results from matches and mismatches between allele and probe sequences in allelic discrimination assays (Livak *et al.*, 1995; Livak *et al.*, 1999).

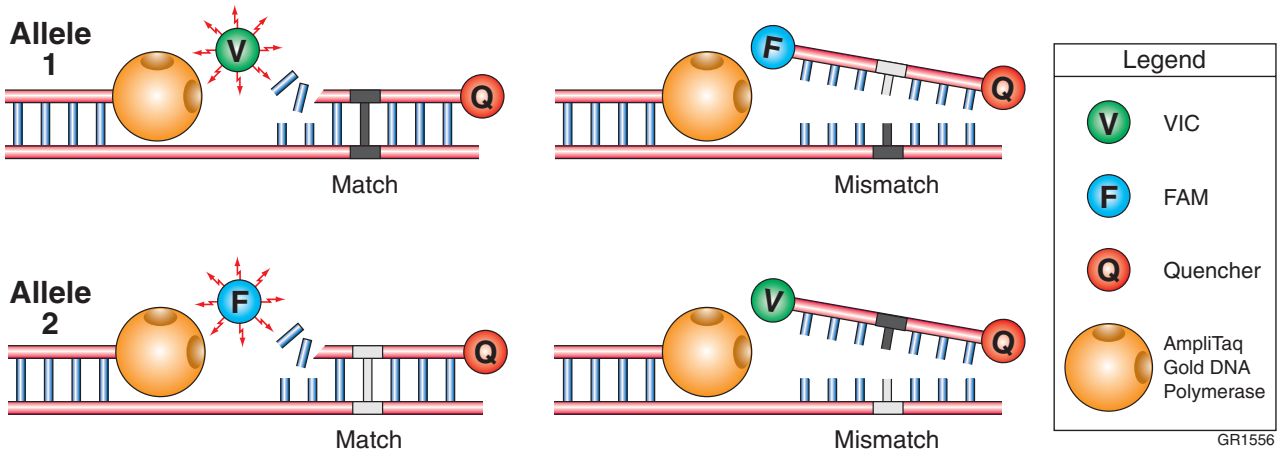


Figure 4-1 Results from matches and mismatches between allele and probe sequences in allelic discrimination assays

The table below summarizes the possible results of the allelic discrimination assays example shown above.

A substantial increase in…	Indicates…
VIC fluorescence only	homozygosity for Allele 1.
FAM fluorescence only	homozygosity for Allele 2.
both fluorescent signals	heterozygosity.

Purchasing an Applied Biosystems Preformulated or Custom-Designed Allelic Discrimination Assay

Assays-on-Demand SNP Genotyping Products

Product Description

Assays-on-Demand™ SNP Genotyping Products provide the widest collection of biologically informative, predesigned, quality-tested, and validated assays for human SNPs that are ready to use on an ABI PRISM Sequence Detection System.

Assays were designed using Applied Biosystems powerful bioinformatics pipeline and software, as well as genomic information from Celera Genomics and public databases. Each assay was validated on 90 genomic DNA samples to ensure the highest quality assay and to provide allele frequency information.

Assays-on-Demand SNP Genotyping Products provide optimized assays for genotyping single-nucleotide polymorphisms (SNPs). The products use the 5′ fluorogenic nuclease assay for amplifying and detecting specific SNP alleles in purified genomic DNA samples. Each assay allows you to genotype individuals for a specific SNP.

Visit the Applied Biosystems web site to view the available products Assays-on-Demand SNP Genotyping Products (PN 4331183). See "How to Obtain Support" on page xi.

For more information about ordering Assays-on-Demand SNP Genotyping Products, contact your Applied Biosystems representative.

Product Properties

The following are properties of Assays-on-Demand SNP Genotyping Products:

- All Assays-on-Demand SNP Genotyping Products are designed and optimized to work with the TaqMan Universal PCR Master Mix, No AmpErase UNG, using the same universal thermal cycling conditions. This facilitates the workflow for all throughput requirements and in any SNP genotyping study.

  Note: The assays can be used with TaqMan Universal PCR Master Mix, which includes AmpErase UNG.

- The assays require only three components:
  - 1 to 20 ng of purified genomic DNA sample
  - 20× SNP Genotyping Assay Mix (specific for each polymorphism)
  - 2× TaqMan Universal PCR Master Mix, No AmpErase® UNG
- The assays require only one amplification step and an endpoint reading to obtain results.

Assay Contents

Each SNP genotyping assay consists of:

- One tube containing 20× SNP Genotyping Assay Mix sufficient for 750 µL reactions
- CD-ROM containing assay information and PDFs of the protocol and product insert

About SNP Genotyping Assay Mix

The SNP Genotyping Assay Mix contains:

- Sequence-specific forward and reverse primers to amplify the SNP of interest
- Two TaqMan MGB probes:
  - One probe labeled with VIC dye detects the Allele 1 sequence
  - One probe labeled with FAM dye detects the Allele 2 sequence

About the Assay Information

The assay information consists of:

- Genomic information about the SNP, including the chromosomal location, allele frequency, and context sequence
- Information about the packaging of each assay tube, including the location in the plate rack and the 2-D bar code

Assays-by-Design Service

If a particular SNP assay of interest is not available as an Assays-on-Demand product, you can use Applied Biosystems Assays-by-Design℠ service (see Chapter 1). The Assays-by-Design service is an assay development service that designs, synthesizes, formulates, and delivers analytically quality-controlled primer and probe for gene expression assays based on sequence information submitted by the customer. For more information, contact your Applied Biosystems representative.

Designing Your Own Allelic Assay

Design and Optimization Steps

This section discusses the Applied Biosystems Assay Design Guidelines developed specifically for allelic discrimination assays. Use these guidelines only when designing your own assays.

These guidelines contain the following important steps:

- Designing probes and primers using Primer Express® software
- Selecting the appropriate reagent configuration
- Using universal thermal cycling parameters
- Using default primer and probe concentrations

IMPORTANT! These steps provide a rapid and reliable system for assay design and optimization only when used in their entirety. In order to achieve the highest level of success, use the system as a whole. Many of the individual components are interdependent.

Conclusions

The Applied Biosystems Assay Design Guidelines enable allelic discrimination assays to be designed and optimized rapidly and efficiently. Since thousands of assays have been developed this way, the following conclusion can be made:

> To attain a highly reproducible and sensitive assay, you can use 900 nM primers, a 200 nM probe, and 1 to 20 ng of genomic DNA.

Probe Design Using Primer Express Software

The Primer Express software uses a set of default parameters to automatically select primer and probe sets.

The probe design guidelines for allelic discrimination assays are discussed here. After choosing probes based on the guidelines below, you design the primer. For information, see “Primer Design Using Primer Express Software” on page 4-8.

TaqMan MGB Probes

The TaqMan MGB probes are two modified conventional TaqMan probes:

- One probe matches the Allele 1 sequence
- One probe matches the Allele 2 sequence

Table 4-2 below summarizes the TaqMan MGB probe features.

Table 4-2 Taqman MGB probe features

Probe	5′ Label	3′ Label	Other Features
TaqMan MGB	FAM or VIC dye	Nonfluorescent quencher	minor groove-binder

When to Use TaqMan MGB Probes

Applied Biosystems recommends the general use of TaqMan MGB probes for allelic discrimination assays:

- To achieve estimated T_m values of 65 to 67 °C using probes shorter than 20 nucleotides
- To obtain greater differences in T_m values between matched and mismatched probes than with conventional TaqMan probes
- To obtain more precise measurements of dye contributions

Allelic Discrimination Probe Design Guidelines

IMPORTANT! When designing probes, it is important to consider probes from both strands.

- To label allelic discrimination probes, use VIC or FAM dyes.
- Avoid probes with G residue at the 5′ end of the probe. A G residue adjacent to the reporter dye will quench the reporter fluorescence, even after cleavage.
- Select probes using Primer Express software (estimated T_m of 65 to 67 °C).
- Make TaqMan MGB probes as short as possible without being shorter than 13 nucleotides.
- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
- Position the polymorphic site in the central third of the probe.

Note: The polymorphic site can be shifted toward the 3′ end to meet the above guidelines, however, the site must be located more than two nucleotides upstream from the 3′ terminus.

Figure 4-2 illustrates the placement of a polymorphism in an example probe (N = Nucleotide).

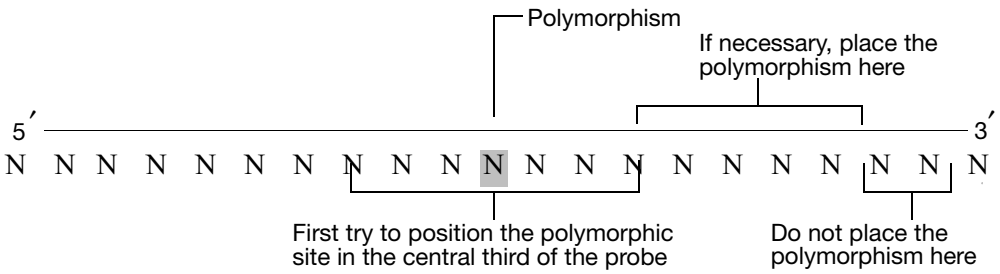

Polymorphism
If necessary, place the polymorphism here
5′
3′
N N N N N N N N N N N N N N N N N N N N
First try to position the polymorphic site in the central third of the probe
Do not place the polymorphism here

Figure 4-2 Polymorphism placement in an example probe

Primer Design Using Primer Express Software

The Primer Express software uses a set of default parameters to automatically select primer and probe sets.

The primer design guidelines for allelic discrimination assays are discussed here. For more information, refer to Primer Express Software Version 2.0 Users Manual (PN 4329500A). After selecting probes for the assay (see page 4-6), choose primers based on the guidelines below.

Note: Primer Express software is designed for universal assay conditions. Changing assay conditions or Master Mix may result in suboptimal performance.

Primer Design Guidelines

If you follow the guidelines below, the amplicons should be 50 to 150 basepairs. By limiting the parameters for amplicon design (such as amplicon size), it is possible to run all reactions with a single reaction buffer (such as TaqMan Universal PCR Master Mix) and a single thermal cycling protocol.

- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more should be avoided.
- The T_m of the primers should be 58 to 60 °C.
- Keep the G/C content within 30 to 80%.
- Make sure the last five nucleotides at the 3′ end contain no more than two G/C residues.
- Place the forward and reverse primers as close as possible to the probe without overlapping it.

Selecting the Appropriate Reagent Configuration

There are several TaqMan chemistry kits available for allelic discrimination assays. The reagent configuration you use depends on your particular assay.

Assays Containing TaqMan MGB Probes

The reagent configurations listed in Table 4-3 are recommended for allelic discrimination assays. These contain the TaqMan MGB probes.

Table 4-3 Reagent configurations for allelic discrimination assays

Product	Reagent Configuration	Part Number
TaqMan	TaqMan Pre-Developed Assay Reagents for Allelic Discrimination	Please see our web site for part numbers (Keyword: PDAR). See “How to Obtain Support” on page xi.
	TaqMan Universal PCR Master Mix, No AmpErase UNG	4324018
Assays-on-Demand	Assays-on-Demand SNP Genotyping Products	4331183
Assays-by-Design	Assays-by-Design Service for SNP Assays	Please see our web site for part numbers (Keyword: genomic assays). See “How to Obtain Support” on page xi.

Quantify the amount of genomic DNA in samples before using Assays-On-Demand SNP Genotyping Products. Generate a standard curve using the DNA Kit (PN 401970) and the RNase P gene primers and probe provided in the TaqMan RNase P Detection Reagents Kit (PN 4316831).

Note: TaqMan RNase P Detection Reagents Kit uses a TaqMan probe with TAMRA dye as the quencher.

Using the Universal Thermal Cycling Parameters

Allelic discrimination assays designed using Applied Biosystems Assay Design Guidelines can be run using the same universal thermal cycling parameters. This eliminates any optimization of the thermal cycling parameters and means that multiple assays can be run on the same plate without sacrificing performance.

Thermal Cycling Parameters

The thermal cycling parameters listed in Table 4-4 are recommended for allelic discrimination assays.

Table 4-4 Thermal cycling parameters for allelic discrimination assays

Times and Temperatures			
Initial Step		**PCR (Each of 40 Cycles)**	
AmpErase UNG Activation[a]	**AmpliTaq Gold DNA Polymerase Activation**	**Melt**	**Anneal/Extend**
HOLD		CYCLE	
2 min @ 50 °C	10 min @ 95 °C	15 sec @ 92 °C	1 min @ 60 °C

a. This step is necessary only if you are using Universal Master Mix with UNG

Note: These conditions are specific for TaqMan Universal PCR Master Mix

Plus/Minus Assays 5

This chapter covers:

About Plus/Minus Assays Using an IPC 5-2
Purchasing the Applied Biosystems TaqMan Exogenous IPC Reagents Kit 5-4

About Plus/Minus Assays Using an IPC

What Is a Plus/Minus Assay?

A Plus/Minus Assay is an endpoint assay that indicates the presence or absence of a specific target sequence in a sample. The actual amount of target is not determined.

Example

A Plus/Minus Assay might be used to determine if the bacteria *Salmonella* is present in hamburger meat. The results will simply show if *Salmonella* is or is not there; the amount of the bacteria present will not be determined.

What Is an IPC?

An internal positive control (IPC) is used in Plus/Minus Assays to monitor the PCR. Plus/Minus Assays can be accomplished without an IPC, however, the IPC ensures that a failed PCR is not mistaken for a negative test result.

The IPC consists of a template and a probe that is added to each well of a reaction plate. Applied Biosystems has developed the TaqMan® Exogenous Internal Positive Control Reagents kit for use in Plus/Minus Assays. These reagents, in conjunction with your target, identify samples that are positive or negative for a specific target sequence. The kit distinguishes between two types of negative reactions:

- Samples identified as negative because they lack the target sequence
- Samples identified as negative because of the presence of a PCR inhibitor

Note: For more detailed information, see "Purchasing the Applied Biosystems TaqMan Exogenous IPC Reagents Kit" on page 5-4.

Instruments

Plus/Minus Assays using an IPC can be used with the following Sequence Detection System (SDS) instruments:

- ABI PRISM® 7000 Real Time PCR System (7000 System)
- Applied Biosystems 7300 Real Time PCR System (7300 System)
- Applied Biosystems 7500 Real Time PCR System (7500 System)
- ABI PRISM® 7700 Sequence Detection System (7700 System)
- Applied Biosystems 7900HT Real Time PCR System (7900 System)

Note: The GeneAmp® 5700 Sequence Detection System (5700 System) cannot be used for Plus/Minus Assays using an IPC.

These instruments are used to measure the increase of reporter fluorescence following PCR. Reporter signals are normalized to the emission of a passive reference, as follows:

$$R_{n\,(TT)} = \frac{\text{Emission Intensity of Target Template Sequence}}{\text{Emission Intensity of Passive Reference}}$$

$$R_{n\,(IPC)} = \frac{\text{Emission Intensity of Internal Positive Control}}{\text{Emission Intensity of Passive Reference}}$$

Chemistry

Plus/Minus Assays using an IPC can be used with fluorogenic 5′ nuclease chemistry (also known as TaqMan chemistry). For information, see "TaqMan Probe-Based Chemistry" on page 2-3.

Note: The SYBR® Green I dye chemistry is not supported for Plus/Minus Assays using an IPC.

Terms Used in Plus/Minus Analysis

Terms commonly used in plus/minus analysis are defined in the table below.

Table 5-1 Terms Used in Plus/Minus Analysis

Term	Definition
Internal positive control (IPC)	A second TaqMan® probe and primer set added to the plate to identify well failure to amplify. Provides a means of determining amplification failure that might give a false negative signal.
NTC No template control (NTC)	A sample that does not contain template. The NTC shows background signal and is used as the negative control. Provides a means of measuring contamination that might give a false positive signal.
Nucleic acid target (also called "target template")	Nucleotide sequence that you want to detect
Unknown sample (also called sample of interest)	The sample for which you want to determine the presence or absence of a specific target

How Plus/Minus Assays Work

Plus/Minus Assays begin by aliquoting the following to each well of a plate: PCR master mix, primers, and fluorogenic probes constructed for the target nucleic acid sequence. Test samples are then added to the plate and loaded into a thermal cycler for thermal cycling.

During the PCR, the fluorogenic probes anneal specifically to the complementary target sequence between the forward and reverse primer sites on the template DNA. Then during extension, AmpliTaq Gold® DNA polymerase cleaves the hybridized probes in each sample containing the target. The cleavage of each matched probe separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter.

After thermal cycling, the plate is run on an SDS instrument, which reads the fluorescence generated during the PCR amplification. The fluorescent signals, measured by the SDS software, can determine the presence or absence of the target nucleic acid in each sample on the plate.

Incorporating an IPC

An IPC is a second TaqMan probe and primer set added to the plate that targets a low-copy, constitutive nucleic acid. If a well does not exhibit amplification, the SDS software uses the positive signal from the IPC to confirm that the well failed to amplify because of a lack of template, rather than a pipetting error.

The configuration of samples on a plus/minus assay plate is slightly different when using an IPC. For plates containing an IPC, primers, probe, and template are pipetted with the target assay to all unknown wells on the plate. In addition to the NTC and unknown samples, a positive control for the IPC is also arrayed on the plate. This group of wells (IPC+) contains IPC template, IPC primers and probe, target primers and probe, but no target template.

Purchasing the Applied Biosystems TaqMan Exogenous IPC Reagents Kit

The Applied Biosystems TaqMan Exogenous Internal Positive Control Reagents kit contains a pre-optimized internal positive control (IPC) that can be spiked into samples to distinguish true target negatives from PCR failure due to inhibition.

Kit Features

The kit is designed to:

- Distinguish types of negative results:
  - A negative call for the target sequence and positive call for the IPC indicates that no target sequence is present.
  - A negative call for the target sequence and negative call for the IPC suggests PCR inhibition.
- Avoid amplification of endogenous genes
- Permit co-amplification of the IPC and the target sequence without compromising amplification of the target sequence
- Perform optimally with the TaqMan Universal PCR Master Mix

Amplifying the IPC and Target in the Same Tube

By using the TaqMan Exogenous IPC Reagents, a low-copy target DNA can be amplified in the same tube with the IPC. Although the target and IPC DNAs may differ in initial copy number, the amplification efficiency of the target reaction is not compromised. This is achieved by limiting the concentration of IPC primers in the PCR reaction.

In the PCR reaction, the IPC is detected using a VIC-dye-labeled probe and the target template is detected using a FAM dye labeled probe.

Endpoint Detection and Post-PCR Plate Read

The TaqMan Exogenous IPC Reagents are designed for endpoint (plate read) assays only. Endpoint detection collects fluorescence data after PCR is complete.

Available Kits

Note: The part numbers listed in Table 5-2 are for 200 reactions. See Appendix C for a list of available kit sizes. The reagent configurations listed below are recommended for Plus/Minus Assays.

Table 5-2 Reagent configurations for plus/minus assays

Reagent Configuration	Part Number
TaqMan Exogenous Internal Positive Control Reagents with TaqMan Universal PCR Master Mix (with VIC® dye)	4308320
TaqMan Exogenous Internal Positive Control Reagents **Note:** If you are using this kit, you will need to purchase one of the core reagents separately, as described below. TaqMan Universal PCR Master Mix, 4304437 TaqMan PCR Core Reagents Kit, N808-0228	4308323

Troubleshooting 6

This chapter covers:

Troubleshooting Quantification Assays 6-2
Troubleshooting Allelic Discrimination Assays 6-6

Troubleshooting Quantification Assays

Using SDS Software to Diagnose Irregular Data

When faced with irregular data, you can use the SDS software to diagnose some chemistry- and instrument-related problems. Figure 6-1 on page 6-6 contains a summary of checks to verify the integrity of your run data and to help you begin troubleshooting potential problems.

Table 6-1 Troubleshooting analyzed run data: quantification assays

Analysis View/Description	Look For
Raw Data Plot Displays the composite raw fluorescence signal (not normalized) for the selected wells during each cycle of PCR.	Signal tightness and uniformity: Do the raw spectra signals from replicate groups and controls exhibit similar spectral 'profiles'? If not, the plate or sample block could be contaminated.
	Characteristic signal shape: Do the samples peak at the expected wavelengths? For example, samples containing only FAM™ dye-labeled TaqMan® probes should not produce raw fluorescence in the wavelength of a VIC® dye component. A signal present in wells that do not contain the dye could indicate that the sample, master mix, or well contains contaminants.
	Characteristic signal growth: As you drag the bar through the PCR cycles, do you observe growth as expected? Absent growth curves may indicate a pipetting error (well lacks template) or no amplification.
	Signal Plateaus: Do any of the signals plateau? Signal plateaus or saturation can be an indication that a well contains too much template or fluorescent signal. The 7700 instrument saturates at 32,500 fluorescent units. The 7900HT instrument saturates at 66,000 fluorescent units
Multicomponent Plot Displays a plot of normalized multicomponent data from a single well of a real-time run. The plot displays the component dye signals that contribute to the composite signal for the well.	Correct dyes displayed: Does the plot display all dyes as expected? The presence of an unexpected dye may be the result of an error in detector setup, such as assigning the wrong reporter or quencher dye.
	ROX™ dye fluorescence level: Does the ROX dye signal fluoresce below the reporter dyes? If not, the lack of reporter fluorescence may be caused by an absence of probe in the well (a pipetting error). A drop in ROX dye fluorescence level can be caused by non-homogenous mix, evaporation, primer/ROX dye interaction, cover not seated properly. **Note:** A slight dip in ROX dye may occur after Cycle 35. This is normal.
	Reporter dye fluorescence level: Does the reporter dye signal fluoresce above background? The background signal is a measure of ambient fluorescence. If a dye fails to fluoresce above the background, it is a strong indication that the well is missing probes labeled with the dye (well does not contain probe, PCR master mix, or both).
	MSE Level: The MSE (mean squared error) is a mathematical representation of how accurately the multicomponented data fits the raw data. The higher the MSE value, the greater the deviation between the multicomponented data and the raw data. When using the 7700 and 7900HT systems, ensure that the plate is set up correctly and the detectors are labeled.

Table 6-1 Troubleshooting analyzed run data: quantification assays *(continued)*

Analysis View/Description	Look For
Amplification Plot Displays data from real-time runs after signal normalization and multicomponent analysis. It contains the tools for setting the baseline and threshold cycle (C_T) values for the run.	Correct baseline and threshold settings: Are the baseline and threshold values set correctly? Identify the components of the amplification curve. Determine the cycle for which amplification first begins to be distinquishable from the noise. Set the baseline so that the endpoint is 1to 2 cycles before this point. Identify the components of the amplification curve and set the threshold so that it is: • Above the background • Below the plateaued and linear regions • Within the geometric phase of the amplification curve
	Irregular amplification: Do all samples appear to have amplified normally, with a smooth amplification plot free of sharp spikes or dips? The three phases of the amplification curve should be clearly visible in from each well.
	Outlying amplification: When the run data is viewed in the C_T vs. Well Position plot, do replicate wells amplify comparably? Wells producing C_T values that differ significantly from the average for the associated replicate wells may be considered outliers. If a plate produces nonuniformity between replicates, some samples on the plate could have evaporated. Check the seal of the optical adhesive cover for leaks.

Troubleshooting Chemistry Problems

Chemistry problems are often the cause of abnormal results. For example, a curve shaped like an upside down U indicates that too much template is present. Table 6-2 describes several chemistry problems that may be encountered when running Quantification Assays.

Table 6-2 Troubleshooting chemistry problems: quantification assays

Observation	Possible Cause(s)	Recommended Action
Poor amplification of target	Poor quality template	Check each template preparation by agarose electrophoresis to determine its purity, ensuring that only one product is formed.
	Poor RT conversion to cDNA	• Check the RNA sample for degradation. • Input RNA could be too concentrated or too dilute. Make sure serial dilutions of template RNA from original stock and repeat RT PCR. • Ensure RT PCR setup has been performed under the appropriate conditions to avoid premature cDNA synthesis.

Table 6-2 Troubleshooting chemistry problems: quantification assays *(continued)*

Observation	Possible Cause(s)	Recommended Action
Poor amplification of target in multiplex assay	Control is too abundant and is outcompeting the target	Optimization of the primer and probe concentrations needs to be performed. Primer concentrations of the more abundant control need to be limited, while concentrations of the target need to be optimized. See *User Bulletin #5 ABI PRISM® 7700 Sequence Detection System SUBJECT: Multiplex PCR with* TaqMan® *VIC® Probes* (PN 4306236) or refer to "Primer Limiting in Multiplex Assays" on page 3-23 of this document for procedures on primer limiting.
Poor amplification of target in multiplex assay (continued)	Probe degradation	Mix and aliquot the probe and primers into single-use aliquots to prevent degradation by freeze-thaw cycles.
Amplification of the target in the NTCs	Contamination of NTCs	Verify that this is true amplification by inspecting the multicomponent view for cleavage of the TaqMan probe. Repeat the assay using new components for NTC reaction mix.
Decrease in fluorescence of the passive reference dye	Precipitation or degradation in the TaqMan buffers	When using the TaqMan PCR Core Reagent kit, be sure to mix the tubes well.
		Use TaqMan Universal PCR Master Mix (PN 4304437). Be sure to mix thoroughly to produce a homogeneous solution.
		Make sure the kits have been stored properly, per instructions on the packaging, and have not expired.
	Interactions between primers and probe with the passive reference dye, that cannot be compensated for by the baseline subtraction	Redesign the primers and/or probe.
Standard curve: poor slope **Note:** A slope value of –3.32 is equal to approximately 100% efficiency.	Assay design guidelines not followed	Follow guidelines precisely. Optimize assay probe concentrations.
	Incorrect dilutions	Redilute the samples. Ensure pipettes are calibrated.
	Inhibitors present in the reaction	Verify extraction method and reprecipitate DNA or RNA.
	Improper reaction conditions (protocols must be followed precisely)	Follow Applied Biosystems recommended thermal profile.

Table 6-2 Troubleshooting chemistry problems: quantification assays *(continued)*

Observation	Possible Cause(s)	Recommended Action
Standard curve: bad correlation coefficient **Note:** The best correlation coefficient is 1.0.	Incorrect baseline and threshold settings	Verify settings according to the user guide specific to your system.
	Improper pipetting	Check calibration of the pipettes. Pipette more than 5 µL of sample.
	Incorrect dilutions	Redilute the samples.

Troubleshooting Allelic Discrimination Assays

Troubleshooting Run Data

DNA Concentration Effects

Observation: Scattering of data points

Possible Causes: Very low DNA concentrations or variable concentrations.

Figure 6-1 shows the benefits of using relatively high DNA concentrations. Using relatively high DNA concentrations yields large ΔRn.

As can be seen in Figure 6-2 on page 6-7, at 1 ng, 5 ng, and 20 ng, within a DNA amount the cluster is tight and easily scorable. The data presented in Figure 6-2 was generated by running the samples at low to high concentrations. If all samples have relatively high (at least 1 ng) DNA concentration, the clusters are scorable. When samples have very low DNA concentrations, such as 0.01 and 0.1, clusters become diffuse, making scoring difficult.

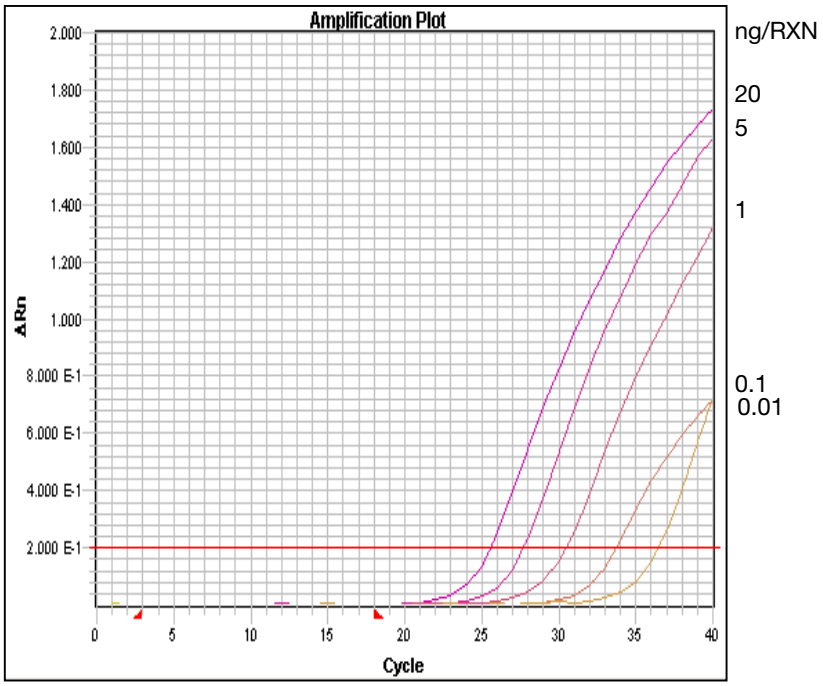


Figure 6-1 Effects of DNA concentration on ΔRn

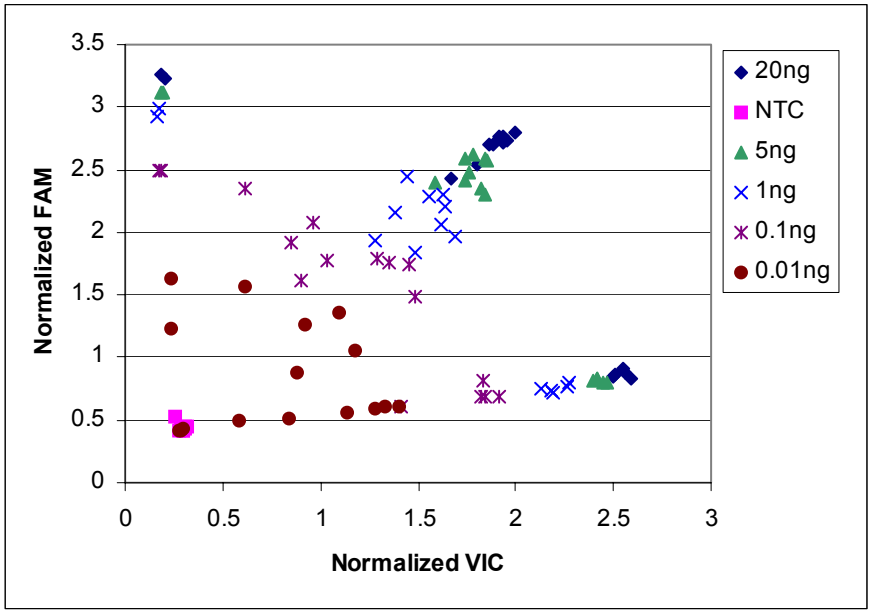


Figure 6-2 Effects of different DNA concentrations

Recommended Action: Verify that samples all have relatively high concentrations.

Troubleshooting Analyzed Run Data

When faced with irregular data, you can use the SDS software to diagnose some chemistry- and instrument-related problems. Table 6-3 contains a summary of checks to verify the integrity of your run data and to help you begin troubleshooting potential problems.

Table 6-3 Troubleshooting analyzed run data: allelic discrimination assays

Analysis View/Description	Look For	Recommended Action
Raw Data Displays the composite fluorescence signal (not normalized) for the selected wells during each cycle of the PCR.	• Signal tightness and uniformity: Do the raw spectra signals from replicate groups and controls exhibit similar spectral profiles? If not, the plate or sample block could be contaminated. • Characteristic signal shape: Do the samples peak at the expected wavelengths? • Signal plateaus: Do any of the signals plateau? Signal plateaus or saturation can be an indication that a well contains too much template or fluorescent signal.	Clean the contaminated block and generate new samples. Repeat the experiment.

Formulas

A

Comparative C_T Method for Relative Quantification

Formula

The amount of target, normalized to an endogenous control and relative to a calibrator, is given by:

$$2^{-\Delta\Delta C_T}$$

Derivation of the Formula

The equation that describes the exponential amplification of PCR is:

$$X_n = X_o \times (1 + E_X)^n$$

where:

X_n	=	number of target molecules at cycle n
X_o	=	initial number of target molecules
E_X	=	efficiency of target amplification
n	=	number of cycles

The threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. Thus,

$$X_T = X_o \times (1 + E_X)^{C_{T,\,X}} = K_X$$

where:

X_T	=	threshold number of target molecules
$C_{T,X}$	=	threshold cycle for target amplification
K_X	=	constant

A similar equation for the endogenous control reaction is:

$$R_T = R_o \times (1 + E_R)^{C_{T,\,R}} = K_R$$

where:

R_T	=	threshold number of reference molecules
R_o	=	initial number of reference molecules
E_R	=	efficiency of reference amplification
$C_{T, R}$	=	threshold cycle for reference amplification
K_R	=	constant

Dividing X_T by R_T gives the following expression:

$$\frac{X_T}{R_T} = \frac{X_o \times (1 + E_X)^{C_{T,\,X}}}{R_o \times (1 + E_R)^{C_{T,\,R}}} = \frac{K_X}{K_R} = K$$

The exact values of X_T and R_T depend on a number of factors, including:

- Reporter dye used in the probe
- Sequence context effects on the fluorescence properties of the probe
- Efficiency of probe cleavage
- Purity of the probe
- Setting of the fluorescence threshold.

Therefore, the constant K does not have to be equal to 1.

Assuming efficiencies of the target and the reference are the same:

$$E_X = E_R = E$$

$$\frac{X_o}{R_o} \times (1 + E)^{C_{T,\,X} - C_{T,\,R}} = K$$

OR

$$X_N \times (1 + E)^{\Delta C_T} = K$$

where:

X_N	=	X_o/R_o, the normalized amount of target
ΔC_T	=	$C_{T,X}$ - $C_{T,R}$, the difference in threshold cycles for target and reference

Rearranging gives the following expression:

$$X_N = K \times (1 + E)^{-\Delta C_T}$$

The final step is to divide the X_N for any sample (q) by the X_N for the calibrator (cb):

$$\frac{X_{N,\,q}}{X_{N,\,cb}} = \frac{K \times (1 + E)^{-\Delta C_{T,\,q}}}{K \times (1 + E)^{-\Delta C_{T,\,cb}}} = (1 + E)^{-\Delta\Delta C_T}$$

where:

$\Delta\Delta C_T$	=	$\Delta C_{T,q} - \Delta C_{T,cb}$

For amplicons designed and optimized according to Applied Biosystems Assay Design Guidelines (amplicon size < 150 bp), the efficiency is close to 1. Therefore, the amount of target, normalized to an endogenous control and relative to a calibrator, is given by:

$$2^{-\Delta\Delta C_T}$$

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B

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Part Numbers

C

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Sequence Detection Instruments

Part Number	Instrument
4329002	ABI PRISM® 7900HT Real Time PCR System with 384-Well Block Module and Automation Accessory
4329004	ABI PRISM® 7900HT Real Time PCR System with 96-Well Block Module and Automation Accessory
4329001	ABI PRISM® 7900HT Real Time PCR System with 384-Well Block Module
4329003	ABI PRISM® 7900HT Real Time PCR System with 96-Well Block Module
4329007	ABI PRISM® 7900HT Real Time PCR System Automation Accessory Upgrade
4331406	ABI PRISM® 7900HT Real Time PCR System 384-Well Block Upgrade Kit
4331405	ABI PRISM® 7900HT Real Time PCR System 96-Well Block Upgrade Kit
4330087	ABI PRISM® 7000 Sequence Detection System
4349042	Applied Biosystems 7300 Real Time PCR system (without computer)
4349142	Applied Biosystems 7300 Real Time PCR system (with laptop computer)
4349143	Applied Biosystems 7300 Real Time PCR system (with tower computer)
4349043	Applied Biosystems 7500 Real Time PCR system (without computer)
4349144	Applied Biosystems 7500 Real Time PCR system (with laptop computer)
4349145	Applied Biosystems 7500 Real Time PCR system (with tower computer)

Sequence Detection Kits

Part Number	Kit	Instrument
4307266	TaqMan® Cytokine Gene Expression Plate 1 with TaqMan® Universal PCR Master Mix and Control Total RNA Two MicroAmp® Optical 96-well Reaction Plates pre-loaded with TaqMan primers and probes for 12 human cytokine targets (replicates of eight) and the 18S Ribosomal RNA endogenous control (in all 96 wells). TaqMan primer and probe concentrations are optimized for multiplex PCR utilizing FAM™ and VIC® dyes. Configuration includes TaqMan Universal PCR Master Mix, MicroAmp® Optical Caps, Control Total RNA (Human), and Protocol.	ABI PRISM® 7700 and 7000 Sequence Detection Systems and Applied Biosystems 7900HT Real Time PCR System
4307265	TaqMan® Cytokine Gene Expression Plate 1 with TaqMan® Universal PCR Master Mix Two MicroAmp Optical 96-well Reaction Plates pre-loaded with TaqMan primers and probes for 12 human cytokine targets (replicates of eight) and the 18S Ribosomal RNA endogenous control (in all 96 wells). TaqMan primer and probe concentrations are optimized for multiplex PCR utilizing FAM and VIC dyes. Configuration includes TaqMan Universal PCR Master Mix and MicroAmp Optical Caps.	ABI PRISM® 7700 and 7000 Sequence Detection Systems and Applied Biosystems 7900HT Real Time PCR System
4306744	TaqMan® Cytokine Gene Expression Plate 1 Protocol	—
4309920	TaqMan® Human Endogenous Control Plate with TaqMan® Universal PCR Master Mix and Control Total RNA Two MicroAmp Optical 96-well Reaction Plates pre-loaded with TaqMan primers and probes for 11 human endogenous control targets and an internal positive control (IPC) in replicates of eight. TaqMan primer and probe concentrations are optimized for 50µl reactions and utilize VIC dye. Configuration includes TaqMan Universal PCR Master Mix, MicroAmp Optical Caps, Control Total RNA (Human), and Protocol.	ABI PRISM® 7700 and 7000 Sequence Detection Systems and Applied Biosystems 7900HT Real Time PCR System
4309921	TaqMan® Human Endogenous Control Plate with TaqMan® Universal PCR Master Mix Two MicroAmp Optical 96-well Reaction Plates pre-loaded with TaqMan primers and probes for 11 human endogenous control targets and an internal positive control (IPC) in replicates of eight. TaqMan primer and probe concentrations are optimized for 50µl reactions and utilize VIC dye. Configuration includes TaqMan Universal PCR Master Mix and MicroAmp Optical Caps.	ABI PRISM® 7700 and 7000 Sequence Detection Systems and Applied Biosystems 7900HT Real Time PCR System
4308134	TaqMan® Human Endogenous Control Plate Protocol	—

Sequence Detection PCR Reagent Kits

Part Number	Kit	Number of 50-µL Reactions
4304437	TaqMan® Universal PCR Master Mix Supplied at a 2× concentration. The mix is optimized for TaqMan reactions and contains AmpliTaq Gold® DNA Polymerase, AmpErase® UNG, dNTPs with dUTP, Passive Reference 1, and optimized buffer components. One 5-mL vial in each box.	200
4318157	TaqMan® Universal PCR Master Mix Ten 5-mL vials in each box.	2000
4305719	TaqMan® Universal PCR Master Mix 10-Pack Ten boxes of PN 4304437.	2000
4326708	50 ml, TaqMan® Universal PCR Master Mix	2000
4324018	TaqMan® Universal PCR Master Mix, No AmpErase® UNG Supplied at a 2× concentration. The mix is optimized for TaqMan reactions and contains AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference 1, and optimized buffer components. One 5-mL vial in each box.	200
4324020	TaqMan® Universal PCR Master Mix, No AmpErase® UNG 10-Pack Ten 5-mL vials in each box.	2000
4326614	TaqMan® Universal PCR Master Mix, No AmpErase® UNG 50 ml	2000
4304449	TaqMan® Universal PCR Master Mix Protocol	—
N808-0228	TaqMan® PCR Core Reagents Kit 250 Units AmpliTaq Gold® DNA Polymerase, 100 Units AmpEase® UNG, dUTP, dATP, dCTP, dGTP, 10× TaqMan Buffer A, 25 mM $MgCl_2$ Solution.	200
4304439	TaqMan® 1000 RXN PCR Core Reagents 1250 Units AmpliTaq Gold® DNA Polymerase, 500 Units AmpEase® UNG, dUTP, dATP, dCTP, dGTP, 10× TaqMan Buffer A, 25 mM $MgCl_2$ Solution.	1000
402930	TaqMan® PCR Core Reagent Kit 10-Pack Ten of PN N808-0228.	2000
402823	TaqMan® PCR Reagent Kit Protocol	—
4304886	SYBR® Green PCR Core Reagents 250 Units AmpliTaq Gold® DNA Polymerase, 100 Units AmpEase® UNG, dNTP Mix with dUTP, 10× SYBR Green PCR Buffer, 25 mM $MgCl_2$ Solution.	200
4306736	10-Pack, SYBR, Green PCR Core Reagents Ten of PN 4304886.	2000

4304965	SYBR® Green PCR Core Reagents Protocol	—
4309155	SYBR® Green PCR Master Mix Supplied at a 2× concentration. The mix is optimized for SYBR Green reactions and contains SYBR Green 1 dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference 1, and optimized buffer components. One 5-mL vial in each box.	200
4312704	SYBR® Green PCR Master Mix 10-Pack Ten of PN 4309155.	2000
4334973	SYBR® Green PCR Master Mix	2000
4310251	SYBR® Green PCR Master Mix Protocol Combined protocol for SYBR Green PCR Master Mix and SYBR Green RT-PCR Reagents.	—

Sequence Detection RT-PCR Kits

Part Number	Kit	Number of 50-µL Reactions
4309169	TaqMan® One-Step RT-PCR Master Mix Reagents Kit Vial1: AmpliTaq Gold® DNA Polymerase mix (2×) is optimized for TaqMan reactions and contains AmpliTaq Gold DNA Polymerase, dNTPs with dUTP, Passive Reference 1, and optimized buffer components. Vial 2: RT enzyme mix (40×) contains MultiScribe Reverse Transcriptase and RNase Inhibitor.	200
4313803	TaqMan® One-Step RT-PCR Master Mix Reagents Kit 10-Pack Ten of PN 4309169	2000
4310299	TaqMan® One-Step RT-PCR Master Mix Reagents Kit Protocol	—
N808-0232	TaqMan® Gold RT-PCR Reagents without controls TaqMan PCR Core Reagents Kit (N808-0228), TaqMan Reverse Transcriptase Reagents (N808-0234).	200
4304133	TaqMan® Gold RT-PCR Reagents without controls 10-Pack Ten of PN N808-0232	2000
402876	TaqMan® Gold RT-PCR Protocol	—
N808-0234	TaqMan® Reverse Transcriptase Reagents MultiScribe Reverse Tanscriptase, RNase Inhibitor, dNTP Mixture, Oligo $d(T)_{16}$, Random Hexamers, 10× RT Buffer, $MgCl_2$ Solution.	200
4304134	TaqMan® Reverse Transcriptase Reagents 10-Pack Ten of PN N808-0234	2000
N808-0236	TaqMan® EZ RT-PCR Core Reagents 1000 Units r*Tth* DNA Polymerase, 100 Units AmpErase UNG, dUTP, dATP, dCTP, dGTP, 5× TaqMan EZ Buffer, 25 mM $Mn(OAc)_2$.	200
403028	TaqMan® EZ RT-PCR Core Reagents 10-Pack Ten of PN N808-0236	2000
402877	TaqMan® EZ RT-PCR Kit Protocol	—
4310179	SYBR® Green RT-PCR Reagents SYBR Green PCR Master Mix (4309155), TaqMan Reverse Transcriptase Reagents (N808-0234).	200
4310251	SYBR® Green PCR Master Mix Protocol Combined protocol for SYBR Green PCR Master Mix and SYBR Green RT-PCR Reagents.	—
4322171	High Capacity cDNA Archive Kit Random primers, Optimized RT Buffer, dNTPs and MultiScribe MULV/RNase Inhibitor mix for the conversion of up to 10 µg of total RNA in a single 100-µL reaction to single-stranded cDNA.	—
4322169	High Capacity cDNA Archive Kit Protocol	—

Sequence Detection Reaction Kits (with Controls)

Part Number	Kit	Number of Reactions
N808-0230	TaqMan® PCR Reagent Kit TaqMan® PCR Core Reagents Kit, B-actin control reagents, and protocol.	200
N808-0233	TaqMan® Gold RT-PCR Reagents with controls TaqMan® PCR Core Reagent Kit, TaqMan® Reverse Transcriptase Reagents, TaqMan® GAPDH Control Reagents, and protocol.	200
N808-0235	TaqMan® EZ RT-PCR Kit 1000 Units r*Tth* DNA Polymerase, 100 Units AmpErase® UNG, dUTP, dATP, dCTP, dGTP, TaqMan® EZ Buffer, $Mn(OAc)_2$, 100 GAPDH control reactions, and protocol.	200

Sequence Detection Control Reagents

Part Number	Kit	Number of Reactions
4308329	TaqMan® Ribosomal RNA Control Reagents (VIC® Dye) Human Control RNA, rRNA Probe (VIC), rRNA Forward Primer, and rRNA Reverse Primer.	1000
4308310	TaqMan® Ribosomal RNA Control Reagents Protocol (VIC® Dye)	—
4316844	TaqMan® RNase P Control Reagents (VIC® Dye) 20× primer and probe (VIC) mix and Human Genomic Control DNA.	1000
4308313	TaqMan® Rodent GAPDH Control Reagents (VIC® Dye) Rodent Control RNA, rodent GAPDH Probe (VIC), rodent GAPDH Forward Primer, and rodent GAPDH Reverse Primer.	1000
4308318	TaqMan® Rodent GAPDH Control Reagents Protocol (VIC® Dye)	—
402869	TaqMan® GAPDH Control Reagents (Human) Human Control RNA, GAPDH Probe (JOE™), GAPDH Forward Primer, and GAPDH Reverse Primer.	100
401846	TaqMan® β-actin Detection Reagents	100
401970	TaqMan® DNA Template Reagents	—
4308323	TaqMan® Exogenous Internal Positive Control Reagents (VIC® Dye) 10× exogenous IPC primer and probe (VIC) mix, 10× exogenous IPC blocking reagent, and 50× exogenous IPC target. Kit represents an IPC assay (optimized with TaqMan® Universal PCR Master Mix) to be used with custom + / - assays.	200
4308321	5-Pack, TaqMan® Exogenous IPC Reagents (VIC® Dye) Five of PN 4308323	1000
4308320	TaqMan® Exogenous Internal Positive Control Reagents (VIC® Dye) with TaqMan® Universal PCR Master Mix (PN 4308323 and 4304437)	200
4308335	TaqMan® Exogenous Internal Positive Control Reagents Protocol (VIC® Dye)	—
4316831	TaqMan® RNase P Detection Reagents Kit 20× primer and probe (FAM™) mix and Human Genomic Control DNA.	100
4310982	TaqMan® RNase P Instrument Verification Plate One ABI PRISM® Optical 96-well Reaction Plate pre-loaded and sealed with TaqMan® primers and probe to detect and quantitate genomic copies of the human RNase P gene.	One 96-Well Plate
4323306	TaqMan® RNase P 384-Well Instrument Verification Plate One ABI PRISM® Optical 384-well Reaction Plate preloaded and sealed with complete TaqMan® primers and probe to detect and quantitate genomic copies of the human RNase P gene.	One 384-Well Plate

Sequence Detection Reagent Components

Part Number	Kits	Quantity
4304441	TaqMan® 1000 RXN Gold with Buffer A Pack 1250 Units AmpliTaq Gold® DNA Polymerase, 10× TaqMan® Buffer A, 25 mM $MgCl_2$ Solution.	1000 Reactions
4305822	Sequence Detection Systems Spectral Calibration Kit	
4311235	MultiScribe™ Reverse Transcriptase, 100 ml (400 Rxn @ 10 µL each, 40 Rxn @ 100 µL)	5000 Units
N808-0119	RNase Inhibitor	2000 Units
N808-0260	dNTP Mixture (10 mM)	1 mL
N808-0128	Oligo d(T)16 (50 µM)	0.1 mL
N808-0127	Random Hexamers (50 µM)	0.1 mL
4307281	Control Total RNA (Human)	100 µL (50 ng/µL)
N808-0096	AmpErase® Uracil N-glycosylase (UNG)	100 µL (1 unit/µL)
4312660	Control Total DNA (Human)	2 tubes, each 100 µL (10 ng/µL)
402929	20% Glycerol Solution, Molecular Biology Grade	100 mL

Sequence Detection Calibration Kits

Part Number	Kit	Quantity
4305822	Sequence Detection Systems Spectral Calibration Kit	—
4323977	Sequence Detection Systems 384-Well Spectral Calibration Kit Two ABI PRISM® Optical 384-Well Reaction Plates. One preloaded and sealed Background plate and one preloaded and sealed Spectral Calibration plate with eight separate dye standards (FAM™, JOE™, NED™, ROX™, SYBR® Green, TAMRA™, TET™, VIC®).	Two 384-Well Plates
4328639	ABI PRISM® 7900HT Sequence Detection Systems 96-Well Spectral Calibration Kit Three ABI PRISM® Optical 96-Well Reaction Plates: one preloaded and sealed Background plate and two preloaded and sealed Spectral Calibration plates containing eight separate dye standards (FAM™, JOE™, NED™, ROX™, SYBR® Green, TAMRA™, TET™, VIC®).	Three 96-well Plates
4328895	ABI PRISM® 7000 Sequence Detection Systems Spectral Calibration Kit Eight ABI PRISM® Optical 96-Well Reaction Plates: one preloaded and sealed Background plate and seven preloaded and sealed Spectral Calibration plates containing seven separate dye standards (FAM™, JOE™, NED™, ROX™, SYBR® Green, TAMRA™, VIC®).	Eight 96-Well Plates

Sequence Detection Disposables

Part Number	Item	Quantity
4313663	ABI PRISM™ Optical Adhesive Cover Starter Kit ABI PRISM™ Optical Adhesive Covers (quantity 20), Applicator (quantity 1), ABI PRISM™ Optical Cover Compression Pad (quantity 1).	20 Covers/Pkg
4311971	ABI PRISM™ Optical Adhesive Covers	100 Covers/Pkg
4314320	ABI PRISM™ Optical Adhesive Covers and ABI PRISM™ 96-Well Optical Reaction Plate with Barcode (code 128) PN 4311971 and 5× ABI PRISM™ Optical 96-Well Reaction Plates (PN 4306737) .	100 Covers 100 Plates/Pkg
4312639	ABI PRISM™ Optical Cover Compression Pads	5 Pads/Pkg
4333183	Adhesive Seal Applicators	5/Pkg
4309849	ABI PRISM™ 384-Well Clear Optical Reaction Plate with Barcode (code 128)	50 Plates/Pkg
4326270	ABI PRISM™ 384-Well Clear Optical Reaction Plate with Barcode (code 128),10-Pack 10× (PN 4309849) ABI PRISM™ 384-Well Clear Optical Reaction Plate with Barcode (code 128).	500 Plates/Pkg
4306737	ABI PRISM™ 96-Well Optical Reaction Plate with Barcode (code 128)	20 Plates/Pkg
4326659	ABI PRISM™ 96-Well Optical Reaction Plate with Barcode (code 128), 25-Pack 25× (PN 4306737) ABI PRISM™ 96-Well Optical Reaction Plate with Barcode (code 128).	500 Plates/Pkg
N801-0560	MicroAmp® Optical 96-Well Reaction Plate Not Barcoded.	10 Plates/Pkg
403012	MicroAmp® Optical 96-Well Reaction Plates and ABI PRISM™ Optical Caps PN 4306737 and 4323032.	20 Plates 2400 Caps/Pkg
4323032	ABI PRISM™ Optical Caps, 8 Caps/Strip Flat Caps.	300 Strips/Pkg 2400 Caps/Pkg
4312063	MicroAmp® Splash Free Support Base for 96-Well Reaction Plates	10 Bases/Pkg
4316567	ABI PRISM™ Optical Tubes, 8 Tubes/Strip For use on ABI PRISM® 7000, 7700 and GeneAmp® 5700 Sequence Detection Systems only.	125 Strips/Pkg 1000 Tubes/Pkg
403081	MicroAmp® 96-Well Tray/Retainer Sets For use with single or strip optical tubes on the ABI PRISM® 7000, 7700 and GeneAmp® 5700 Sequence Detection Systems.	10 Sets

N801-0933	MicroAmp® Optical Tubes For use on ABI PRISM® 7000, 7700 and GeneAmp® 5700 Sequence Detection Systems only.	2000/Pkg
4330015	ABI PRISM™ Cap Installing Tool Used for removing MicroAmp® Caps or ABI PRISM™ Optical Caps from the MicroAmp® plate or ABI PRISM™ 96-Well Optical plate and Tray/Retainer Assemblies.	One/Pkg
433292	ABI PRISM™ Snap-On Compression Pads Snap-On Compression Pad is a compliant cover bonded to a metal frame, and is designed for use on the Applied Biosystems 7900HT Real Time PCR System when using the available automation accessory with 96-well plates.	9/Pkg

Custom Oligonucleotide Synthesis

Part Number	Item	Delivery Time
4316034	TaqMan® MGB Probe 5,000 to 6,000 pmoles 5'-Fluorescent label: 6-FAM™, VIC®, or TET™.	6 to 7 days[a]
4316033	TaqMan® MGB Probe 15,000 to 25,000 pmoles 5'-Fluorescent label: 6-FAM™, VIC®, or TET™.	6 to 7 days[a]
4316032	TaqMan® MGB Probe 50,000 to 100,000 pmoles 5'-Fluorescent label: 6-FAM™, VIC®, or TET™.	6 to 7 days[a]
450025	TaqMan® TAMRA Probe 5,000 to 6,000 pmoles 5'-Fluorescent label: 6-FAM™, VIC®, or TET™.	4 to 5 days[a]
450024	TaqMan® TAMRA Probe 15,000 to 25,000 pmoles 5'-Fluorescent label: 6-FAM™, VIC®, or TET™.	4 to 5 days[a]
450003	TaqMan® TAMRA Probe 50,000 to 100,000 pmoles 5'-Fluorescent label: 6-FAM™, VIC®, or TET™.	4 to 5 days[a]
4304970	Sequence Detection Primers Minimum 4,000 pmoles purified for sequence detection.	3 days[a]
4304971	Sequence Detection Primers Minimum 40,000 pmoles purified for sequence detection.	4 days[a]
4304972	Sequence Detection Primers Minimum 130,000 pmoles purified for sequence detection.	4 days[a]

a. For Custom Oligonucleotide Synthesis, consider the following:
- Delivery includes only business week days, orders to be received by email or online web ordering before 10:00 a.m. PST/PDT.
- Orders received by fax will be entered into the order system on the next business day.
- Quantities are based on an average oligo length of 23mer.

Contact information:

To order online: http://store.appliedbiosystems.com

To order by email: OligosUS@appliedbiosystems.com

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